

EFFECT OF BORON ON THE DIFFERENTIATION OF MESENCHYMAL STEM CELLS

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Boron is an essential microelement in the metabolism of living organisms. However, its role is not well defined yet. It has been shown recently that boron has a positive effect on the differentiation on murine myoblasts and some reports describe boron to be involved on bone mineralization. For that reasons, it is done in this work a research using different concentrations of boron in a material system composed by PLLA (poly lactic-L-acid) as a substrate, in order to verify if boron has also an effect on the differentiation of mesenchymal stem cells to different lineages (myoblast, osteoblast, adipocyte or for the contrary stemness maintenance). With this, it could be favored the regeneration of both damaged muscle tissue and bone tissue, achieving in that way an important progress on tissue engineering, adding to the current techniques just a microelement, boron.

Keywords: boron – differentiation – mesenchymal stem cells – PLLA

Efecto del boro en la diferenciación de células madre mesenquimales

El boro es un microelemento esencial en el metabolismo de los seres vivos, aunque su papel en este no está bien definido. Se ha demostrado recientemente que el boro tiene un efecto positivo en la diferenciación de mioblastos murinos, y algunos estudios describen que el boro puede intervenir en la mineralización del hueso. Por ello, se realiza aquí un estudio empleando diferentes concentraciones de boro y PLLA (ácido-L-poliláctico) como soporte, con el fin de comprobar si el boro tiene también un efecto en la diferenciación de células madre mesenquimales hacia varios linajes diferentes (mioblasto, osteoblasto, adipocito o por el contrario mantenimiento de las células en su estadio indiferenciado). Se podría con ello favorecer la regeneración tanto de tejido muscular como tejido óseo dañado, consiguiendo así un avance importante en la ingeniería de tejidos, añadiendo a las técnicas actuales tan solo un microelemento, el boro.

Palabras clave: boro – diferenciación – células madre mesenquimales – PLLA

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1. Introduction

1.1. Context: tissue engineering

This project is framed on the relatively new field of Tissue Engineering. This field applies the principles of engineering and biology in order to develop biological substitutes that restore, maintain or improve tissue function mainly when a tissue has been damaged or an organ has failed (Berthiaume, Maguire, and Yarmush 2011; Langer and Vacanti 1993). The strategy followed to create a new tissue structure combines three main components:

- a) **Living cells:** these cells can be obtained either from a donor or from a patient. When they are obtained from the patient, they can be mesenchymal stem cells, cells from the tissue that wants to be repaired or induced stem cells.
- b) **Biocompatible materials:** polymers that serve as support and protection for the cells, so they provide an environment similar to the extracellular matrix of their native tissue.
- c) **Biochemical factors:** substances and biomolecules that favor the proliferation and/or differentiation of the cells. They can be used *in vitro* to prepare the cells before implanting them, or they can be loaded into the biocompatible material to be released inside the body.

These three strategies can be used to create a tissue-like structure with the objective of regenerating a damaged tissue or organ. In this project there is a special focus on a biochemical factor acting as a bioactive ion: boron. We performed a novel approach to test the effects of boron on mesenchymal stem cell differentiation. A biocompatible material, poly-lactic acid, is going to be used. This will allow assessing the potential of boron for applications in tissue engineering.

In the following sections they will be developed the main reasons for choosing boron, poly-lactic acid and mesenchymal stem cells on this project.

1.2. Biochemical factor as bioactive ion: boron (B)

In tissue engineering the biochemical factors are substances and molecules that improve the proliferation and/or differentiation of cells. An objective widely pursued in tissue engineering is to favor cell differentiation, in order to obtain cells that will replace the specific damaged tissue. Growth factors, signal molecules and microelements are used for this purpose. The factors used to differentiate mesenchymal stem cells to either bone, muscle or adipose cells will be detailed in section 1.4. The biochemical factors to differentiate each type of cell are selected through a process of both trial and error and through analysis of molecules that have shown potential. Boron is one of these potential factors, as has been shown in numerous studies.

Boron (B) is a metalloid essential for life as a micronutrient. It is usually found in the Earth as boric acid $B(OH)_3$ and in a lesser extent as borate $B(OH)_4^-$ (Zangi 2012). Its relevance and mechanism in plants have been exhaustively described (Blevins and Lukaszewski 1998). In animals and humans, it has been shown to be involved in metabolism and homeostasis regulation (Pizzorno 2015). However, its mechanism of action is not fully understood yet.

1.2.1. Role of boron in physiology

After boron was widely accepted as an essential element in plants, researchers have been studying its relevance in animals. Boron deficiency has been shown to affect negatively the life cycle, with impaired growth, development or maturation. In mammals there is no finding about effects of boron in the life cycle, but it has been shown by various studies of boron depletion-repletion that it is beneficial and probably required for health (Nielsen 2014; Hunt 2012). The 1996 report of the World Health Organization, "Trace elements in human nutrition and health" (World Health Organization 1996), includes boron as a trace element that is probably essential for life.

Boron can be found in human diet in vegetables, fruits, legumes and nuts, ranging from 0.86 to 2.06 mg in a typical serving of the richest food sources (Pizzorno 2015). The metalloid is highly soluble, so it is absorbed through gastrointestinal epithelia and excreted in urine. The concentration of boron in urine is similar to the boron intake, and it has been found that a higher B intake does not increase significantly its plasma levels (Nielsen and Penland 1999; Liao et al. 2011), meaning that there must be a mechanism that regulates efficiently boron homeostasis. It is believed that the body has a storage reserve of boron because more than 21 days are necessary to see changes in humans fed by a low-boron diet. It is important to take into account that in

studies where the boron intake is increased in humans it is possible to see positive changes in the patient, meaning that their daily intake was probably lower than the necessary. World Health Organization recommends 1mg/day, and settles the safe intake range at 1-13mg/day (WORLD HEALTH ORGANIZATION 1996). Since the date of report of the WHO, many studies have confirmed the relevance of boron for human health.

It was firstly suggested that boron can enhance and mimic some effects of estrogen therapy in post-menopausal women, meaning that an increased intake of boron can alleviate the symptoms of post-menopause by preventing calcium loss and bone demineralization. Boron achieves this by reducing calcium and magnesium urinary excretion, increasing serum levels of 17β -estradiol and testosterone (Nielsen, Mullen, and Hunt 1987; Nielsen and Penland 1999). In an early study it was shown that plasma calcium and serum 25-hydroxycholecalciferol (indicator of vitamin D levels) were lower during boron depletion and serum calcitonin and osteocalcin were higher during boron depletion (Nielsen, Mullen, and Gallagher 1990). Due to effect on bioavailability of estradiol and vitamin D, boron is said to affect steroid hormone metabolism in humans. It can be also concluded that boron has a role on mineral metabolism, due to the changes of its effect depending on the concentration of other minerals like magnesium.

Boron has also been found to reduce levels of inflammatory biomarkers such as high-sensitivity C-reactive proteins (CRP) and tumor necrosis factor α (TNF- α) (Naghii et al. 2011). This is of relevance because high levels of CRP are related to increased risk of cardiovascular disease, cancer, obesity and stroke, among other diseases. Other interesting effects of boron in mammals reviewed by Pizzorno (2015) are that it raises levels of antioxidant enzymes, protects against oxidative stress induced by pesticides and heavy-metal toxicity and improves the cognitive performance, as well as the short-term memory in elders.

In studies regarding the action of boron at a molecular rather than physiological level, it has been found to regulate the expression of certain proteins. Dzondo-Gadet et al. (2002) showed that adding 10mM of boron at cell-free systems increased by 6 fold the RNA synthesis of genes coding for proteins involved in angiogenesis (VEGF), wound healing (TGF β) and growth factors (TNF α). They also concluded that boron effect was exerted on a transcriptional level rather than translational. In order to have effect in transcription, it is necessary that B enters the cell. In aqueous solution B is found as multivalent specie poly-hydroxylated. In this state is hydrophilic and medium-sized, reason why it was hypothesized that it passed through the membrane by simple diffusion or through an unspecific ion channel (Zangi 2012). In plants there is a boron

transporter, AtBor1 (Takano et al. 2002), which has a homologous in mammals, NaBC1. In the absence of borate, it transports Na^+ and OH^- through the cell membrane. In the presence of borate, it acts as an electrogenic Na^+ coupled borate co-transporter, very specific for borate and sensitive even at very low concentrations (Park et al. 2004). Later, it was described how dietary supplementation of boron alters expression of NaBC1 mRNA in pigs (Liao et al. 2011). As B is absorbed through the gastrointestinal epithelia, the researchers studied the expression of NaBC1 in cells of this tissue, as well as in kidney, one of the most relevant organs for homeostasis regulation. When supplementing the pig's diet with boron, the expression of NaBC1 mRNA increased a 213% in jejunum and decreased a 35% in kidney. This data suggests that both intestine and kidney regulate B uptake and excretion. Loss of expression of NaBC1 in kidney causes polyuria and an increase in urine of Na, Cl, K and Mg, supporting the theory that states that B is implicated in mineral metabolism.

Silencing of NaBC1 reduces a 50% the incorporation of thymidine in the cell. In their research Park et al. (2004) showed that borate at low concentrations has a mitogenic effect by activating the MAPK pathway through phosphorylation, increasing in that way cell proliferation and growth. The initiation and elongation steps of DNA transcription also require phosphorylation (Dzondo-Gadet et al. 2002). These are the reasons why probably silencing of NaBC1 reduced incorporation of a molecule relevant in cell proliferation and RNA synthesis.

Another explanation for the mechanism of action of boron is defined by its biochemistry. Boric acid forms complexes with hydroxyl groups in cis orientation. These groups are found mainly in sugars as ribose, a component of adenosine. When analyzing by capillary electrophoresis which molecules bound strongly to boron, they were found S-adenosylmethionine (SAM) and diadenosine phosphates (Ralston and Hunt 2001), both molecules that contain adenosine. Around 95% of SAM is used in methylation reactions, influencing the activity of DNA, RNA, proteins, phospholipids, hormones and neurotransmitters. There is a study in rats that backs the hypothesis that part of the boron activity is through SAM formation and/or use (Nielsen 2014). On the other hand, boron also binds strongly to the ubiquitous molecule nicotinamide adenine dinucleotide (NAD⁺), giving strength to the relevance of boron in the molecular level.

1.2.2. Drugs and boron

As seen in the previous section, boron has many functions at the molecular level, which are mainly due to its particular chemical structure. B is very similar to carbon –with one electron less– meaning that it is a good carbon analog. This characteristic is useful for pharmaceuticals

based on enzyme inhibition, particularly of hydrolytic enzymes, due to the change of trigonal to tetrahedral form that occurs both in carbon and boron transition state molecules during enzymatic hydrolysis (Yang, Gao, and Wang 2003). As reviewed in Baker et al. (2009) other enzyme inhibitors containing boron that are being used to treat disease are inhibitors of dipeptidyl peptidase 4 (DPP4) and inhibitors of phosphodiesterase 4 (PDE). One DPP4 inhibitor containing boron, Januvia®, was approved by the FDA for the treatment of type II diabetes, and another one, Talabostat, is in phase III clinical trials to treat non-small cell lung cancer. AN2728 is in phase II clinical trials as PDE inhibitor for the treatment of psoriasis. Additionally, other boron based serine protease inhibitors are being studied as anticoagulants, as they inhibit thrombin or Factor X_{II} in the blood coagulation cascade. TRI50c is in phase III clinical trials as anticoagulant.

One particular use of boron containing molecules is for cancer treatment. Among the first boron-containing compounds approved for human use are drugs that include a non-radioactive isotope of boron-10 for their use in boron neutron-capture therapy (BNCT), to treat brain, head and neck tumors (Soloway et al. 1998). Velcade® is a peptidic proteasome inhibitor to treat multiple myeloma and non-Hodgkin's lymphoma, and it was the first boron based therapeutic in the market (Adams and Kauffman 2004). Boric acid has shown to have antiproliferative effects by decreasing serum prostate specific antigen (Gallardo-Williams et al. 2004), decreasing expression of A-E cyclin (Barranco et al. 2009) and activating ER-β (estrogen receptor), which has an anti-proliferative role (Ohta et al. 2013). One study suggested that boron-rich diets are correlated with lower incidence of cervical cancer, possibly through the serine protease inhibition of HPV oncogene (Korkmaz et al. 2007). Increased boron intake is correlated with decreasing odds of lung cancer with a similar effect compared to hormone replacement therapy, possibly because higher boron intake increases levels of estradiol, which compete for estrogen receptors with PAHs from cigarette smoke (Mahabir et al. 2012).

Boron has also therapeutic effects improving bone condition, wound healing and muscle differentiation. These will be treated in the following section, as they are of particular interest in this work.

1.2.3. Boron in bone regeneration and maintenance

Relevance of boron for osteogenesis was elucidated in studies that demonstrated that its deficiency adversely impacts bone development and regeneration. Chicks deprived from boron decreased their chondrocyte density in the growth plate (Hunt, Herbel, and Idso 1994), the second generation of frogs fed with low boron diet showed abnormal limb development (Fort et

al. 2000) and pigs fed with boron purified diet had lower bending moment, breaking load and tolerance stress on the femur (Armstrong et al. 2000). Rats fed on a low boron diet showed a significant lower femur strength, vertebral trabecular thickness and femur concentrations of copper, iron and magnesium compared to rats fed with 3mg/kg of boron (Nielsen, Stoecker, and Penland 2007; Nielsen and Stoecker 2009). In mice, dietary boron deprivation led to impaired modeling and remodeling of periodontal alveolar bone, since they had a 63%-48% reduction of osteoblast surface and 58%-73% increase in quiescent surface (Alejandro Gorustovich et al. 2008b). The same group described how boron deprivation impaired bone healing, probably because of a reduction in the osteogenesis process (Alejandro Gorustovich et al. 2008a).

Besides proving the adverse effects of low boron diets in animals, these studies have also shown how a boron supplement to the normal diet improved in overall bone strength. Of particular interest are the effects of boron in the regulation of key hormones and mineral metabolism, as it was mentioned in section 1.2.1. Boron supplementation in post-menopausal women reduced calcium and magnesium excretion in urine and increased serum levels of 17 β -estradiol and testosterone (Nielsen, Mullen, and Hunt 1987; Nielsen and Penland 1999). In this studies, as well as in others that show increase of sex hormones in serum (Naghii et al. 2011), it is suggested that it is unlikely that boron has a direct effect on mineral metabolism, because the amount of boron in the diet (3.25mg) was very low compared to the amount calcium, phosphorus and magnesium given (316-870mg). Instead, they suggest that boron regulates mineral metabolism through endocrine mechanisms, due to the significant increased levels of 17 β -estradiol and testosterone in serum, as well as the enhancement of the action of estrogen (Sheng et al. 2001) and vitamin D (Hunt, Herbel, and Idso 1994) on improving bone quality and mineral adsorption.

As described in Hunt et al. (1994), boron supplementation in chicks with inadequate intake of vitamin D alleviates the characteristic adverse effects of vitamin D deficiency. Miljkovic et al. (2004) proposed a mechanism through which boron could increase serum concentration of 25-hydroxyvitamin D and improves its effects. His group suggested that boron suppresses the activity of 24-hydroxylase, a microsomal enzyme responsible for the catabolism of vitamin D, avoiding the degradation of the hormone. The same hypothesis applies to 17- β estradiol, which is catabolized too by a microsomal enzyme. Boron might do this by forming complexes with hydroxyl groups in cis orientation, which happen to be the end product of the hydroxylation of vitamin D and 17- β estradiol. The complex might act as a competitive inhibitor of the microsomal enzyme or as a down-regulator of the enzyme. Vitamin D affects bone health through diverse mechanisms (Sunyecz 2008). In vitamin D deficiency states, the absorption of calcium by the

intestine is decreased, causing increased osteoclast production. Osteoclasts break the bone matrix to release calcium and increase the amount of circulating calcium. Vitamin D directly interacts with receptors in the osteoblasts, increasing their formation. These osteoclasts also break the bone matrix. On the other hand, low serum calcium levels stimulate the parathyroid gland to release parathyroid hormone, which increases renal absorption of calcium and also osteoclast production. If vitamin D deficiency is not corrected, the bone matrix keeps being broken, leading to osteoporosis. Vitamin D deficiency has shown to damage bone, distort marrow sprouts, delay cartilage calcification, decrease calcium and phosphorus absorption, decrease growth and femur calcium concentration (Nielsen 2008).

Regarding the reduction of calcium and magnesium excretion observed in boron supplemented diets, it is important to notice that 60% of human magnesium is found in bone, where it directly affects two regulators of calcium homeostasis: parathyroid hormone (PTH) and calcitriol, the active form of vitamin D. Hypomagnesemia results in an increase of intracellular calcium level, which inhibits the secretion of PTH, resulting in hypocalcemia, diminished reabsorption of calcium in kidney and low serum concentrations of vitamin D. Additionally, the enzyme responsible for production of calcitriol (the most active form of vitamin D), 25-hydroxycholecalciferol-1-hydroxylase, requires magnesium as a cofactor. This results in hypomagnesemia impairing calcitriol production. Hypomagnesemia provokes also insulin resistance, altering the bone mineralization process by increasing glucose concentration, which has been shown to impair calcium deposition in bone. These findings, together with the regulation of hormone metabolism (vitamin D, estrogen and testosterone), explain how boron supplementation can improve bone regeneration and prevent bone loss in humans.

However, more recent studies of the same group with higher sample numbers (Nielsen 2004; Nielsen, Stoecker, and Penland 2007; Nielsen and Stoecker 2009) have shown that boron deprivation does not markedly affect calcium and phosphorus concentration in bone, but it affects the concentration of other mineral elements associated with the differentiation and activity of osteoblasts and osteoclasts, meaning that boron is directly beneficial for bone growth and maintenance by promoting osteoblasts activity and not through affecting bone calcium concentration or hormone homeostasis, even if its regulation may help to prevent bone loss. This provides more background to test how boron affects the *in vitro* differentiation of mesenchymal stem cells to osteoblasts. More support to this hypothesis is found in studies that have shown that boron upregulates the expression of genes related to tissue mineralization, osteoblastic differentiation and bone formation.

The effect of boron in pre-osteoblastic cells (MC3T3) (Hakki, Bozkurt, and Hakki 2010) and bone marrow stromal cells (BMSC) (Ying et al. 2011) was tested. The metalloid remarkably enhanced cell viability, proliferation, mineralization nodules and expression of bone mineralized tissue-associated proteins, particularly collagen type I, osteopontin, bone sialoprotein, osteocalcin, alkaline phosphatase and RunX2, compared to untreated cells. It also increased mRNA expression of bone morphogenetic protein (BMP), specifically BMP4, BMP5 and BMP7. BMP are growth factors that belong to the TGF- β family which induce formation of new cartilage and bone. RunX2 is a transcription factor that stimulates osteoblastic differentiation from mesenchymal stem cells, as well as bone formation and bone maintenance (Franceschi et al. 2008). When its levels are reduced, other genes related to tissue mineralization drop its expression, as bone sialoprotein, osteocalcin and osteopontin.

Finally, several tissue engineering studies have shown beneficial effects of boron for osteoblast regeneration with different biomaterials. In 2003, Gough et al. tested the biocompatibility of PCL (polycaprolactone) and borontrifluoride for craniofacial bone regeneration. They showed improved cell proliferation, differentiation and higher amount of mineralized collagen 1 matrix in PCL scaffolds synthesized with borontrifluoride. Gorustovich et al. (2006) implanted bioactive glass particles modified with boron oxide in rat tibia. Boron increased bone regeneration, thickness and calcium:phosphorous ratio at 15 days. At 30 days post-implantation, boron only improved thickness. They suggest that boron acts in an early stage of osteogenic differentiation. Wu et al. (2011) tested mesoporous bioactive glass scaffolds containing boron for the proliferation and differentiation of human osteoblasts. It was improved the expression of collagen I and RunX2 in the boron-containing scaffolds. These studies show and support the potential of boron in bone tissue engineering.

1.2.4. Boron in myogenic differentiation

In spite of all the evidence that supports the important role of boron in bone tissue regeneration, little is known about its effect on muscle tissue. Boron nitride nanotubes are being used as nanomaterials for biomedicine. However, this is mainly due to its physical-chemical characteristics, tridimensional structure and biocompatibility, not by a special improvement on muscle differentiation (Ciofani et al. 2013). It has been described how tetraphenylboron changes the charge of the surface of the sarcoplasmic reticulum, allowing Ca^{2+} release in skeletal muscle (Liu and Oba 1990; Soler, Fernandez-Belda, and Gomez-Fernandez 1989). However, the release of

Ca^{2+} is responsible for contraction of fully differentiated muscle cells, and does not have a paper in the differentiation.

Only two recent studies have described the effects of boron in muscle differentiation. Apdik et al. (2015) tested the effect of boric acid on myogenic differentiation of human adipose stem cells (hADSC). Just $5\mu\text{g}/\text{mL}$ of boron increased cell viability and were not toxic for the cells. It increased expression of mRNA specific of myocytes in early stages of differentiation, particularly myosin heavy chain, myogenin, desmine and MyoD, being the last one the one with higher fold change compared to the control. However, a higher dose of boron at day 21 decreased the expression of these proteins.

In the field of tissue engineering, Rico et al. (2015) showed the effect of poly-L-lactic acid (PLLA) substrates loaded with borax on the differentiation of murine C2C12 myoblasts, a model for muscle differentiation. The substrates containing borax had a higher percentage of differentiated cells and higher surface of myotube, compared to the standard control with collagen I as substrate. However, the myogenic markers MyoD and myogenin showed no significant improvement between the control and the PLLA substrates loaded with borax. This can be explained by the multistep process of differentiation of muscular cells, being possible that boron acts at an earlier stage than the moment when the analysis of the presence myogenic markers was done.

1.3. Biocompatible material: poly-L-lactic acid

In tissue engineering, it is common the use of polymeric scaffolds or surfaces that serve as support and protection for the cells, having an environment similar to extracellular matrix of their native tissue (Langer and Vacanti 1993). The polymers can be used to induce differentiation of the cells *ex vivo* or can be implanted *in vivo* for tissue regeneration, with or without cells inside it.

Ideal polymers for tissue engineering should be biocompatible and resorbable at a controlled rate which matches cell or tissue growth, should have a suitable chemistry for cell attachment, proliferation and differentiation, as well as mechanical properties to match those of the tissue to regenerate (Hutmacher 2000). Many polymers, natural or synthetic, have been used in tissue engineering. In this study poly-L-lactic acid (PLLA) has been chosen, as it can be considered as an ideal polymer, according to the characteristics mentioned above. It is composed by the L isomer of hydrophobic aliphatic polyester. PLLA has been widely approved by the US Food and Drug Administration (FDA) for direct contact with biological fluids and it is generally recognized as safe.

As Farah et al. (2016) review, PLLA has numerous advantages compared to other biopolymers. PLLA's production is eco-friendly, as it requires less energy to be produced -making it cheaper-, it is obtained from renewable sources and it is biodegradable. It is biocompatible and reabsorbable, meaning that its degradation products are not toxic and do not interfere with the tissue healing. In human body it is hydrolyzed to its constituent α -hydroxy acid, which is incorporated into the tricarboxylic acid cycle and excreted. It has higher processability, so it can be synthesized and shaped using multiple techniques. On the other hand, it has several drawbacks, as a slow degradation rate, low toughness and high hydrophobicity, which worsen cell affinity. However, these problems have been solved by blending PLLA with other polymers.

PLLA has been widely used in biomedical applications that show its versatility: nerve and spinal cord injury regeneration, bioabsorbable screws in ankle, knee and hand; meniscus repair, guided bone regeneration, cardiac regeneration, stents, surgical sutures, dentistry, space filler, plastic and reconstructive surgery, dermal fillers, oncology, drug delivery, etc. (Tyler et al. 2016).

It has been used both for bone and muscle regeneration, including in the Rico et al. (2015) study with boron, where they prove that boron does not affect the properties of PLLA and analyzed its release from the polymer. It is then a suitable material to use as biocompatible substrate in this study. In this case it is not used as scaffold, because it has not been shaped as a tridimensional porous structure, but it is used as a surface easy to synthesize and that is simple to later analyze the cell differentiation, without requiring complex equipment.

1.4. Living cells: mesenchymal stem cells

The key elements in tissue engineering are the living cells that are going to regenerate the damaged tissue. These cells can be derived from donor tissue or progenitor cells (Berthiaume, Maguire, and Yarmush 2011). The donor tissue can be from the patient or from other person, being the last one dangerous for the receptor due to immunological incompatibilities. Stem or progenitor cells have several advantages that make them suitable for tissue engineering: they have a high proliferative capacity and they are pluripotent (they can differentiate into several lineages). Stem cells can be obtained from embryos, placenta, umbilical cord, bone marrow of adults, or they can be induced pluripotent stem cells (iPSC), which can be any type of differentiated cell converted in stem cell.

The cells chosen in this work are the murine embryonic mesenchymal stem cells C3H10T1/2. They have been demonstrated to differentiate into osteoblast, chondrocyte, adipocyte and myoblast, being suitable for this study (Shin et al. 2000). Mesenchymal stem cells (MSCs) have been used in tissue engineering (Ringe et al. 2002) due to their extensive *in vitro* proliferation and their potential to differentiate to multiple non hematopoietic lineages.

MSCs can be differentiated to different lineages by adding specific inductors to culture medium. In this study, the lineages that are induced are osteoblast, myoblast and adipocyte. This last is used as a control for osteoblast differentiation, as bone induction blocks adipogenesis, and vice-versa (Q Chen et al. 2016).

2. Objective

Myogenesis and osteogenesis are vital processes in the life cycle, which can be disrupted due to disease, injuries or aging. Tissue engineering aims at improving muscle and bone regeneration by using biomaterials, cells and biochemical factors that enhance genesis of tissue, to repair damages. Research and progress in developing and discovering any of the strategies of tissue engineering helps to this purpose.

This study is motivated by the research done by our group in the Center for Biomaterials and Tissue Engineering (CBIT), where it is demonstrated that borax loaded PLLA substrates are able to induce muscle cell differentiation and myotube formation. Boron is a promising biochemical inducer, as it has demonstrated extensively its beneficial effects in life, health and muscle and bone differentiation, and hence regeneration. A step forward in the knowledge of how and under which circumstances does boron act can help to improve regenerative medicine.

This work aims at testing the effect of boron in murine mesenchymal stem cells, particularly to demonstrate if it is capable to induce differentiation to osteoblast and myoblast lineages. This will allow knowing if boron acts at early stages of differentiation, before the compromise of cells to any particular lineage. The substrate used is poly-L-lactic acid prepared with a method in which PLLA merely acts as a support for the cells, being boron directly added to the culture medium. The study is qualitative, using fluorescence microscopy to analyze markers and morphology of the cells.

3. Materials and methods

3.1. Experimental design

This study is based on the qualitative analysis of the cells to check their differentiation state. The workflow is the following:

1. Preparation of the **polymeric substrates**
2. Preparation of **cells** and the different culture media
3. **Cell culture** over the polymeric substrates
4. **Immunofluorescence essay** and analysis with fluorescence microscopy OR staining and analysis with inverted optical microscope.
5. **Image treatment** with specialized software

A total of 4 different conditions are tested for each experiment, and there are a total of 9 experiments.

The four **conditions** are the following, each one with three samples:

1. Crystal slide cover (C+, positive control)
2. PLLA
3. PLLA + 2% borax
4. PLLA + 5% borax

The nine **experiments** are defined by different molecular markers that define each type of cell (osteoblast, myoblast, adipocyte and stem cell undifferentiated). To each of the four conditions explained above, either basal medium (negative control) or differentiation medium are used.

1. RunX2 (osteoblast) + basal medium
2. RunX2 (osteoblast) + osteoblast medium
3. OPN (osteoblast) + basal medium
4. OPN (osteoblast) + osteoblast medium
5. α -actinin (myoblast) + basal medium
6. α -actinin (myoblast) + myoblast medium
7. Liposome (adipocyte) + basal medium
8. Liposome (adipocyte) + adipocyte medium
9. Sca1 (stemness) + basal medium → negative control

Given that, for each of the 9 experiments there are 4 conditions tested (positive control, PLLA, PLLA+2% borax, PLLA+5% borax), and for each condition there are 3 samples.

3.2. Polymeric substrate

The PLLA substrates were prepared from a solution of PLLA (Cargill Dow) dissolved in 100 mL of chloroform to obtain a final concentration of 2% (w/v). The materials were prepared using spin coating technique.

Spin coating is a method that allows to deposit a thin layer of the biopolymer (PLLA 2%) over a surface. The surfaces used were crystal slide covers of 12 mm diameter, with a total surface of 2 cm². The slide covers are vacuum dried at room temperature in order to evaporate the excess of solvent. Boron is not released from the polymer, but it is added to the culture medium, with the same concentrations as if it was released from a solvent casting prepared polymer, as described by Rico et al. (2015): major release is produced during the first 3 hours of culture, and the release of borax increases as the concentration of borax in the sample does. At 14 days there is still release of borax from the polymer.

Two solutions of PLLA 2% containing 2% and 5% of borax (sodium tetraborate decahydrate Na₂B₄O₇·10H₂O) (Bórax España S.A) in relation to total mass of polymer were prepared, following the procedure of Rico et al. (2015). These will be added to the culture media depending on the experiment performed.

The substrates were sterilized 30 minutes at UV light, before being used for cell culture.

The two types of substrate were coated during 1h at room temperature with fibronectin (FN) from human plasma (Sigma-Aldrich), using a solution of 20 µg/mL in Dulbecco's phosphate-buffered saline (DPBS).

3.3. Biological material and culture media

The cells used were murine mesenchymal stem cells C3H10T1/2 (ATCC). They were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% Fungizone (Life Technology, Fisher) and 1% penicillin–streptomycin (Lonza) in humidified atmosphere at 37 °C and 5% CO₂. Cells were subcultured before reaching confluency, to obtain the needed concentration of cells before starting differentiation.

According to the nine experiments stated in section 3.1, it is necessary to use four different culture media. It was added either 2% or 5% borax to the culture media, depending on the experiment performed.

- Basal medium: DMEM + 10% FBS + 1% fungizone + 1% penicillin-streptomycin.
 - Borax 2%
 - Borax 5%
- Osteogenesis induction medium: DMEM + 10% FBS + 1% fungizone + 1% penicillin-streptomycin + 50 µg/mL ascorbic acid + 10mM β-glycerophosphate + 0.1 µM dexamethasone.
 - Borax 2%
 - Borax 5%
- Myogenesis induction medium: DMEM + 10% horse serum (HS) + 1% fungizone + 1% penicillin-streptomycin.
 - Borax 2%
 - Borax 5%
- Adipogenesis induction medium: DMEM + 10% FBS + 1% penicillin-streptomycin + 1% Fungizone + 0.5 mM, 3-isobutyl-1-methylxanthine + 60 µM Indometacine + 0.5 µM Hydrocortisone
 - Borax 2%
 - Borax 5%

Once obtained the desired concentration of cells, the cultures were stimulated to differentiate. 20000 cells/cm² were cultured over FN-coated crystal slide cover (C+), PLLA, PLLA-2% borax and PLLA-5% borax, three samples per each one of the substrates. The substrates of 12mm Ø were placed in Nunclon plates of 24 wells. A total of 400 µL of culture media were added, according to the experiment (differentiation medium or basal medium; no borax, 2% borax or 5% borax). Every 3 days, the culture medium was changed. The cultures were kept in humidified atmosphere at 37 °C and 5% CO₂ until analysis of the samples was done.

For OPN, α-actinin, adipogenesis and stemness experiments, the cultures were stopped at day 15. For RunX2, it was stopped at 3 days. For myoblast, osteoblast and stemness, the next step is an immunofluorescent essay. For adipocyte, it is a cell staining.

3.4. Immunofluorescence assay

After the 3 or 15 days, depending on the experiment, the cultures were immunostained with specific markers of their lineage, in order to analyze their morphology and their stage of differentiation.

The immunofluorescence assay is based in the binding of a primary antibody to the chosen molecular marker. Then, a fluorophore-conjugated secondary antibody is added. This binds to the primary antibody, and has fluorescence in a certain wavelength. Depending on the marker that wants to be seen, different primary and secondary antibodies are used.

The general protocol for immunofluorescence begins fixing cells with formaline 4% and then permeabilizing the cells with DPBS/Triton X-100 0.5% during 5 minutes at room temperature. Next, cells are blocked during 1 hour at room temperature with DPBS and goat serum 5% (for RunX2, OPN and Sca1) or horse serum 5% for α -actinin sarcomeric. Cells are washed with DPBS/Triton X-100 0.1%.

Cells are incubated with primary antibody 1 hour at 37 °C or overnight at 4 °C. The primary antibody is different depending on the marker:

- Myoblast: mouse antibody anti mouse α -actinin sarcomeric (Abcam) dilution 1:200 in DPBS/HS 5%.
- Osteoblast:
 - Rabbit antibody anti mouse RunX2 (Abcam) dilution 1:200 in DPBS/GS 5%.
 - Mouse antibody anti mouse OPN (Santa Cruz Biotechnology) dilution 1:200 in DPBS/GS 5%.
- Stemness: rat antibody anti mouse Sca1 (Abcam) dilution 5 μ g/mL in DPBS/GS 5%.

Unbound antibody is washed with DPBS/Triton X-100 0.1%.

Cells are incubated with secondary antibody during 1 hour at 37 °C. The secondary antibody is different depending on the primary antibody used. This is the antibody that gives the fluorescence, and depending on the fluorophore of the antibody, it will emit fluorescence in different wavelengths.

- Myoblast: anti mouse Cy3 conjugated antibody (Jackson Immunoresearch) dilution 1:200 in DPBS/HS 5%.
- Osteoblast:
 - Alexa Fluor 488 goat anti rabbit (Invitrogen) dilution 1:100 in DPBS/GS 5%.

- Alexa Fluor 488 goat anti mouse (Invitrogen) dilution 1:200 in DPBS/GS 5%.
- Stemness: Alexa Fluor 488 goat anti rat (Fisher) dilution 5 μ g/mL in DPBS/GS 5%.

Unbound antibody is washed with DPBS/Triton X-100 0.1%.

The samples are mounted in microscope slide covers, staining the nucleus with Vectashield containing DAPI (Atom) in the case of α -actinin sarcomeric, OPN and Sca1. For RunX2, it is mounted with FluorSave (Calbiochem WVR).

The samples are observed under a fluorescence microscope (Nikon Eclipse 80i). 10 images for each condition (C+, PLLA, PLLA-B2%, PLLA-B5%) and experiment were taken. The channels were used according to the secondary antibody fluorophore:

- α -actinin sarcomeric: DAPI channel (blue) and Cy3 channel (red). Images taken at 10X and 20X magnification.
- RunX2: Alexa Fluor 488 channel (green). Images taken at 20X and 40X magnification.
- OPN: DAPI channel (blue) and Alexa Fluor 488 channel (green). Images taken at 10X and 40X magnification.
- Sca1: DAPI channel (blue) and Alexa Fluor 488 channel (green). Images taken at 10X and 20X magnification.

3.5. Cell staining

After 15 days in adipocyte differentiation culture medium, the cultures are stained with Oil Red O, a fat soluble dye. Cells are equilibrated with isopropanol 60% during 2 minutes at room temperature. Oil Red O solution (1mg/mL Oil Red O (Sigma-Aldrich) + 60% isopropanol + distilled H₂O) is added, 1mL per well, during 15 minutes at room temperature. Cells are washed twice with distilled water. The samples are mounted in microscope slide covers with glycerol. They are observed under inverted optical microscope. 10 images for each condition (C+, PLLA, PLLA-B2%, PLLA-B5%) and experiment were taken, at magnification 20X and 40X.

3.6. Image treatment

The obtained images were treated using the software Fiji (Image J), specialized for scientific image analysis. Images with high background noise were readjusted. The brightness and

contrast were adjusted in order to normalize all the images. As the images obtained from α -actinin sarcomeric, Sca1 and OPN were separated in two channels, the nuclei and the cell, they were merged to have a complete vision of the cell morphology.

It is important to notice that PLLA emitted green fluorescence, so in some cases the images had strong background noise.

|

4. Results and discussion

4.1. Stemness

As a negative control of differentiation, one experiment was a 15-day culture of mesenchymal stem cells in basal culture medium. This allow us to check how an undifferentiated cell should look under the four different conditions (C+, PLLA, PLLA+B2%, PLLA+B5%).

The marker used for the immunofluorescence was Sca1 (stem cells antigen 1). It is a glycosyl phosphatidylinositol-anchored cell surface protein that is commonly used to identify mouse stem cells, including mesenchymal stem cells (Holmes and Stanford 2007). DAPI was used to stain the nucleus.

Figure 1 shows the images of MSC cultured in basal medium, over crystal slide cover (C+) (A and B) and over PLLA (C and D). Under these conditions, the cells are undifferentiated and they cover most of the surface due to their continuous growth. In some cases, the cytoplasm of the cells can't be discriminated due to overlapping between them.

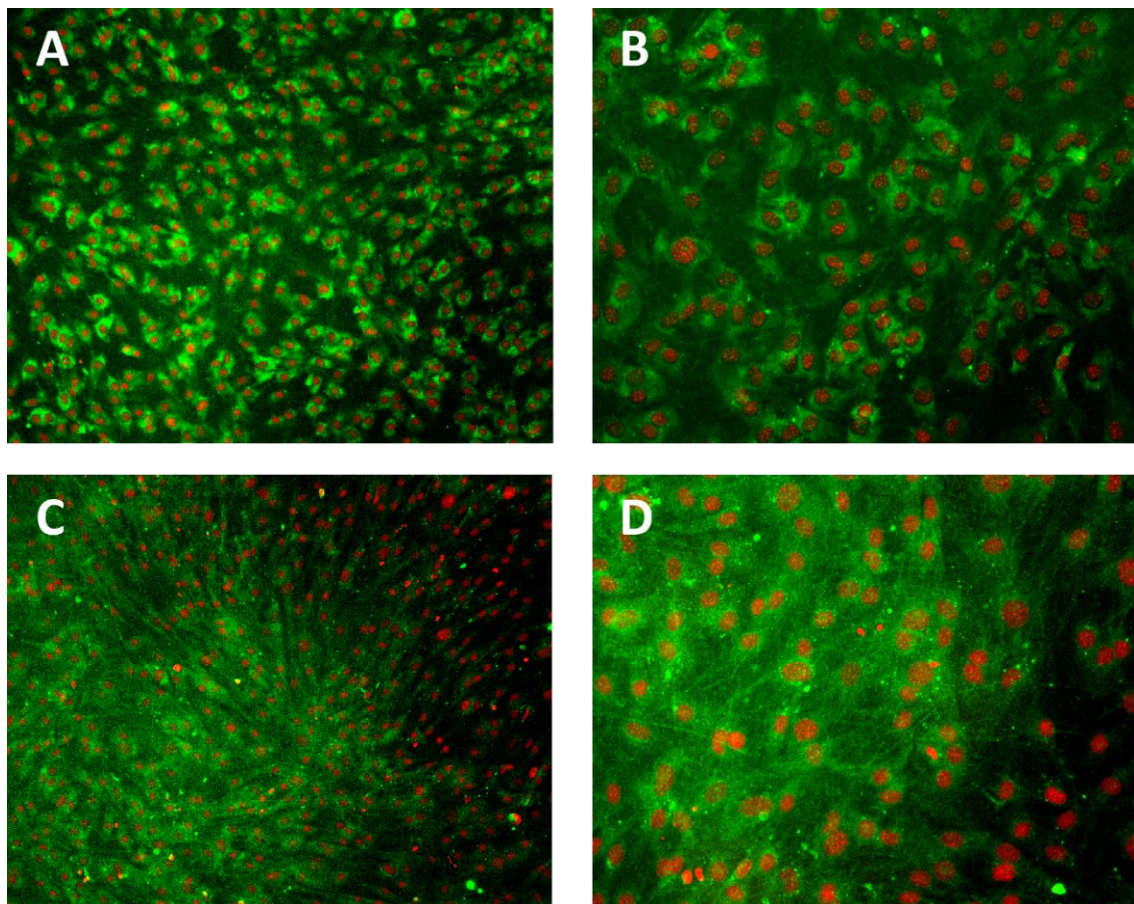


Figure 1. Immunofluorescence of Sca 1. Cell culture of MSC with basal medium. A and B) Culture over crystal slide cover, at 10X and 20X magnification respectively. C and D) Culture over PLLA, at 10X and 20X magnification respectively.

Figure 2 shows the images of MSC cultured over PLLA with basal medium, with 2% boron (A and B) and 5% boron (C and D). Cell density (according to the number of nuclei) does not decrease, supporting the theory that boron is not toxic for the cells. The marker Sca1 seems to be more expressed in these cells. No specific morphology can be observed in these cells, and probably they don't acquire fibroblast-like shape due to the high cell density.

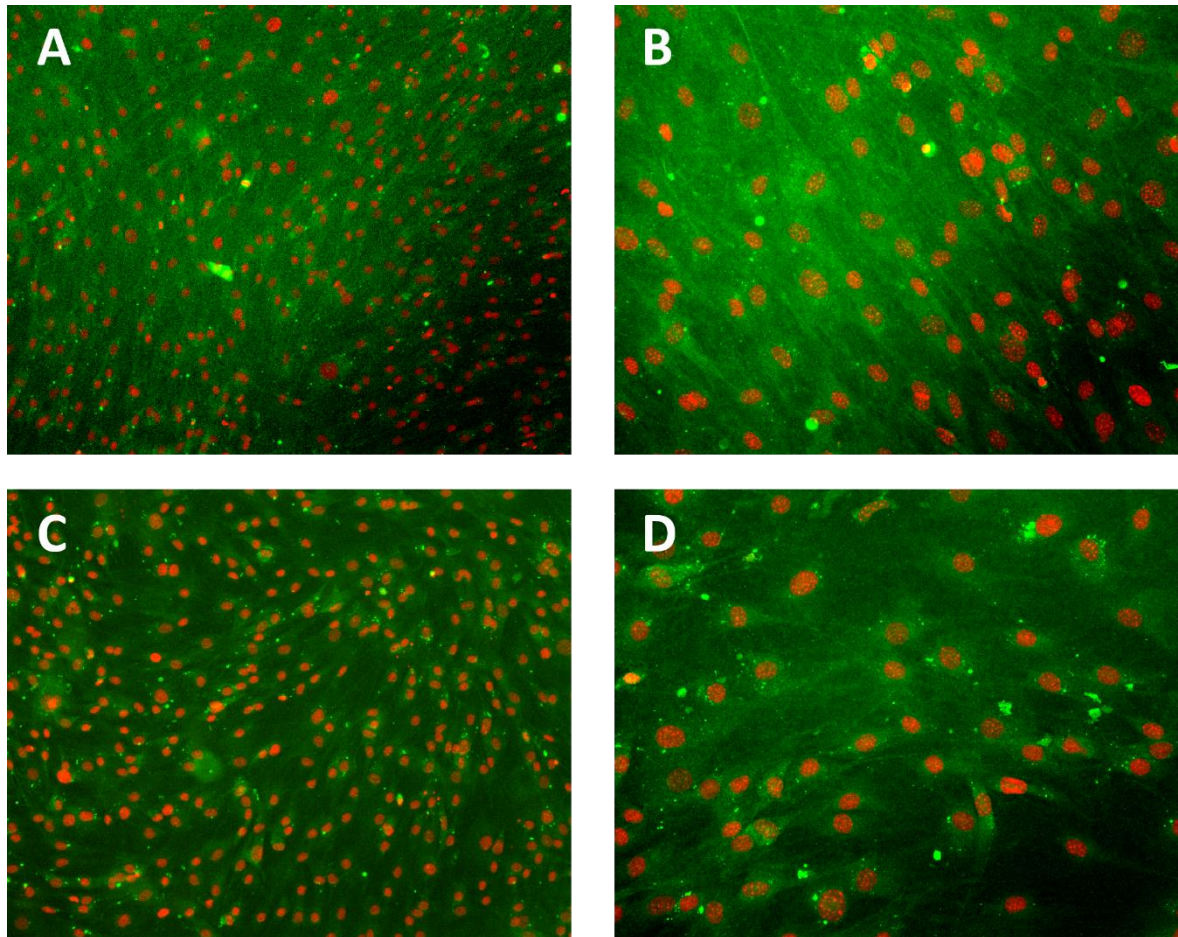


Figure 2. Immunofluorescence of Sca 1. Cell culture of MSC with basal medium over PLLA. A and B) Culture in medium with 2% boron added, at 10X and 20X magnification respectively. C and D) Culture in medium with 5% boron added, at 10X and 20X magnification respectively.

4.2. Osteogenesis

To test the cells for differentiation to the osteoblastic lineage, the markers used were RunX2 and OPN.

RunX2 (runt-related transcription factor) is an essential transcription factor for osteoblast differentiation. It is detected in pre-osteoblasts, and its expression increases in immature osteoblasts (Komori 2010). As it is a transcription factor, it will be found in the nucleus when the cell is differentiating to osteoblast. In these experiments, the nucleus was not stained with DAPI,

so the RunX2 in the nucleus could be seen.

OPN (osteopontin) is a soluble protein that is present intracellularly and as an extracellular structural protein that interacts with cell surface receptors, including integrins (Chen et al. 2014). It is expressed in immature osteoblasts rather in pre-osteoblasts, and it forms part of the extracellular matrix of bone (Komori 2010). The transcription factor RunX2 induces the expression of OPN. DAPI was used to stain the nucleus.

4.2.1. RunX2

These cultures were left during 3 days, presumably enough to induce early differentiation in mesenchymal stem cells.

In figure 3 it is shown the culture of MSC over C+, with basal medium (A and B) and osteogenic medium (C and D). With basal medium the shape of the cells is similar to the shown in figures 1 and 2 of the Sca1 marker, meaning that the cells are undifferentiated. With differentiation medium, the shape changes to a more branched morphology.

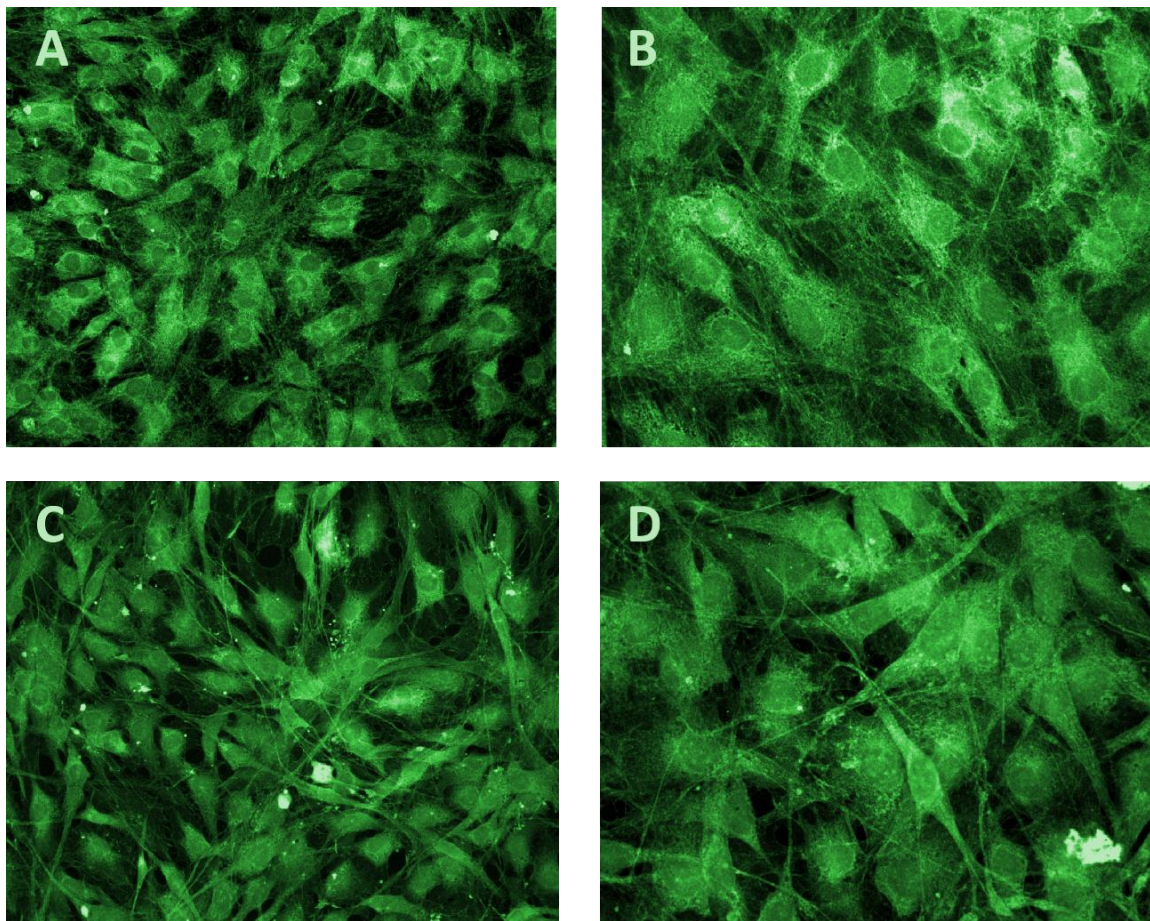


Figure 3. Immunofluorescence of RunX2. Cell culture of MSC over crystal slide cover. A and B) Culture with basal medium, at 20X and 40X magnification respectively. C and D) Culture with osteogenic medium, 20X and 40X magnification respectively.

In none of the cases, however, it is seen a clear location of the RunX2 marker inside the nucleus, where it should be in differentiated cells, so it must be located in the cytoplasm in its inactive form (non-phosphorylated). There exists the possibility that 3 days is a short time for osteoblast differentiation, as the bibliography reviewed used times of 1 week or longer. This means that the transcription factor is being synthesized but it is not being activated.

In figure 4, cells cultured over PLLA substrate, with basal medium (A and B) and osteogenic medium (C and D) show in both cases a more branched morphology. In this case, in the C and D images it can be distinguished the fluorescence of the nuclei (red arrows), signaling the presence of the transcription factor RunX2 in the nucleus. Cell density in C and D is lower. It is possible that the PLLA substrate enhances cell differentiation under differentiation conditions.

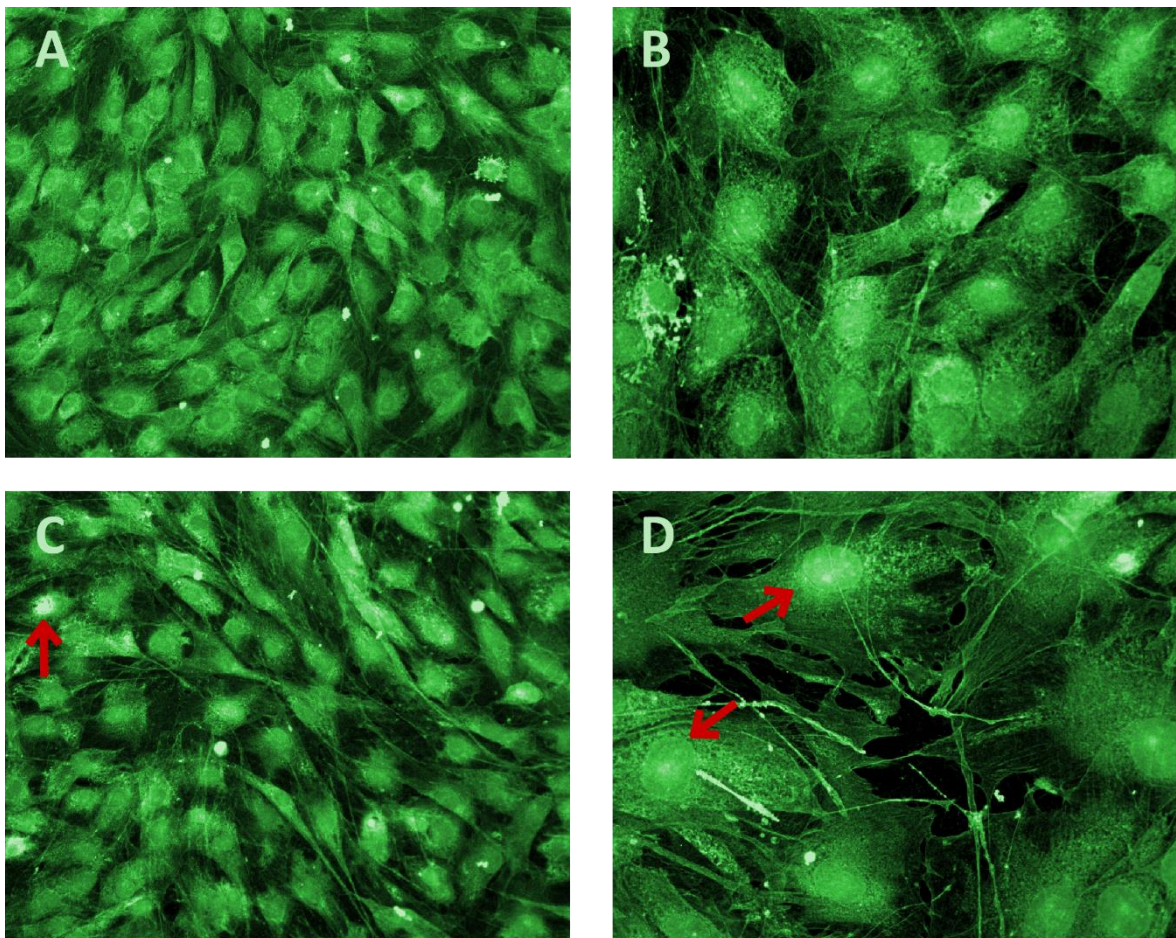


Figure 4. Immunofluorescence of RunX2. Cell culture of MSC over PLLA. A and B) Culture with basal medium, at 20X and 40X magnification respectively. C and D) Culture with osteogenic medium, 20X and 40X magnification respectively.

Figure 5 shows the cells cultured over PLLA substrate with boron 2% in the basal (A and B) and osteogenic (C and D) medium. In this case, it is evident the different morphology when comparing to the images in figure 2 (Sca1, PLLA+B2%), both in basal and osteogenic medium. The nuclei A and B have higher intensity compared to PLLA with basal medium, suggesting that boron can induce differentiation even with basal medium. Cell density in both cases is much lower than in the C+ and PLLA. Cell cycle is stopped during differentiation, so cells do not replicate. This means that a lower cell density is equal to higher differentiation. D and C figures show morphology more similar to osteoblast, as well as nuclei with a more intense fluorescence.

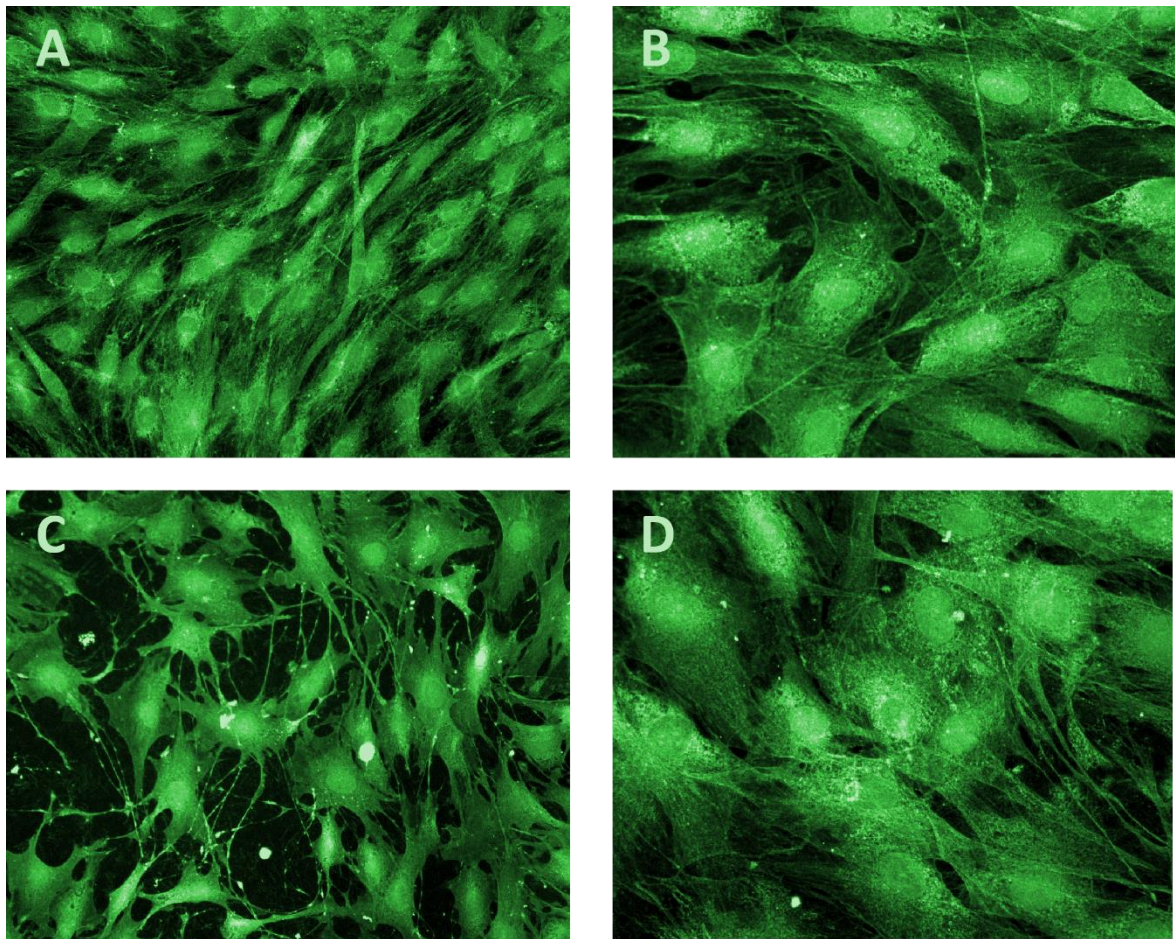


Figure 5. Immunofluorescence of RunX2. Cell culture of MSC over PLLA with 2% boron in medium. A and B) Culture with basal medium, at 20X and 40X magnification respectively. C and D) Culture with osteogenic medium, 20X and 40X magnification respectively.

Figure 6 shows the cells cultured over PLLA substrate with boron 5% in the basal (A and B) and osteogenic (C and D) medium. These images show lower cell density comparing to PLLA+B2%, in basal and osteogenic medium. In both media the cells have a marked pre-osteoblast shape and defined nuclei. Additionally, in figures C and D the nuclei are clearly more intense, meaning that RunX2 is inside. When comparing these images to figure 3C and 3D (osteogenic medium in C+), and taking in account the nuclei intensity in figure 5, it is suggested that even if 3 days may be a short time for osteogenic differentiation, boron induces it, leading to the activation of RunX2.

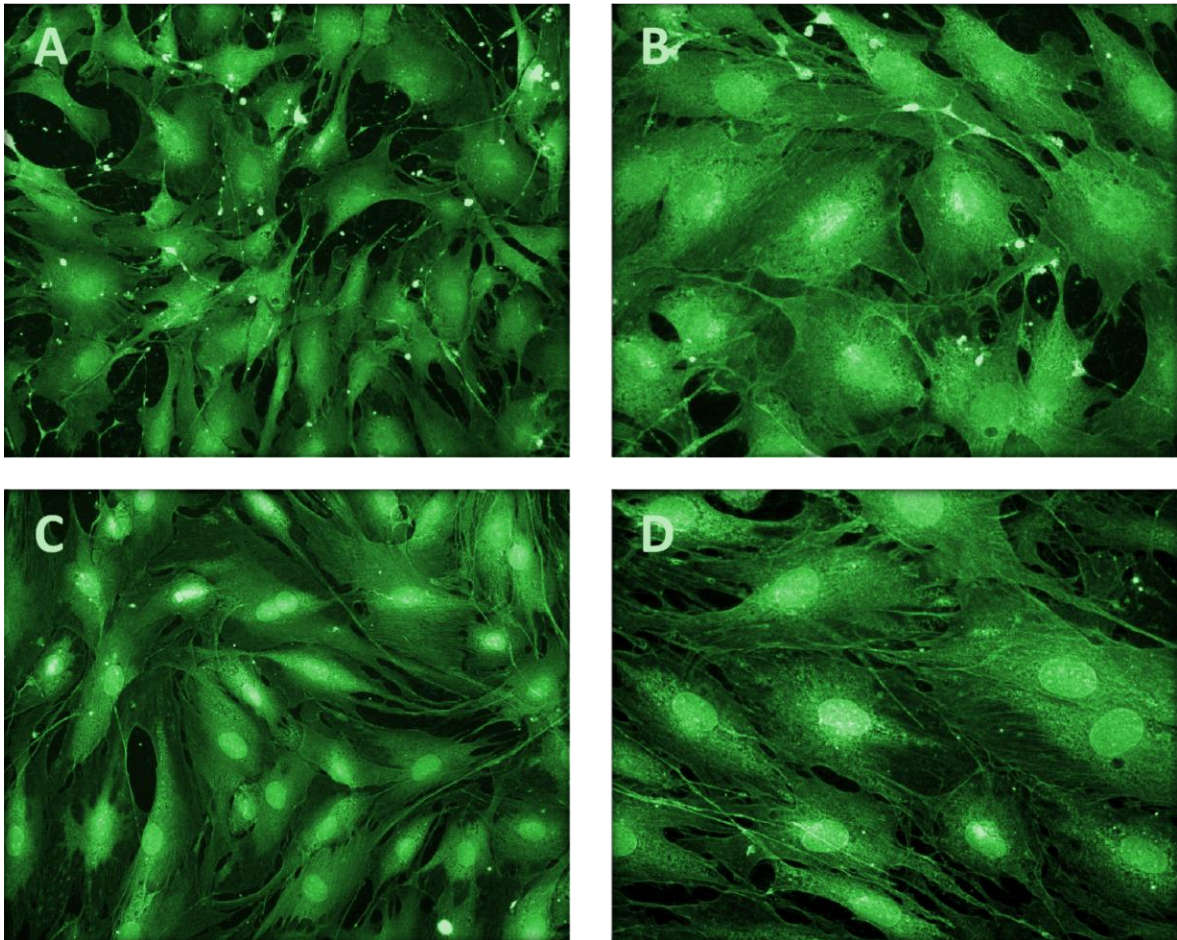


Figure 6. Immunofluorescence of RunX2. Cell culture of MSC over PLLA with 5% boron in medium. A and B) Culture with basal medium, at 20X and 40X magnification respectively. C and D) Culture with osteogenic medium, 20X and 40X magnification respectively.

4.2.2. OPN

These cultures were left during 15 days. Taking in account those 3 days could be few time to differentiate MSC to pre-osteoblasts, it is expected that 15 days are enough for immature osteoblasts, which is the stage when OPN is expressed.

In figure 7 it is shown the culture of MSC over C+, with basal medium (A and B) and osteogenic medium (C and D). The most noticeable difference between basal medium and osteogenic medium is how the cytoplasm is more delimited and there is less background in osteogenic medium. OPN has been found in MSC, being excreted to the medium, because it is used as signaling molecule too (Chen et al. 2014). However, it is upregulated in osteoblasts. It is possible that it is being synthesized and accumulated in the cytoplasm, and its excretion is performed later in the osteogenesis process.

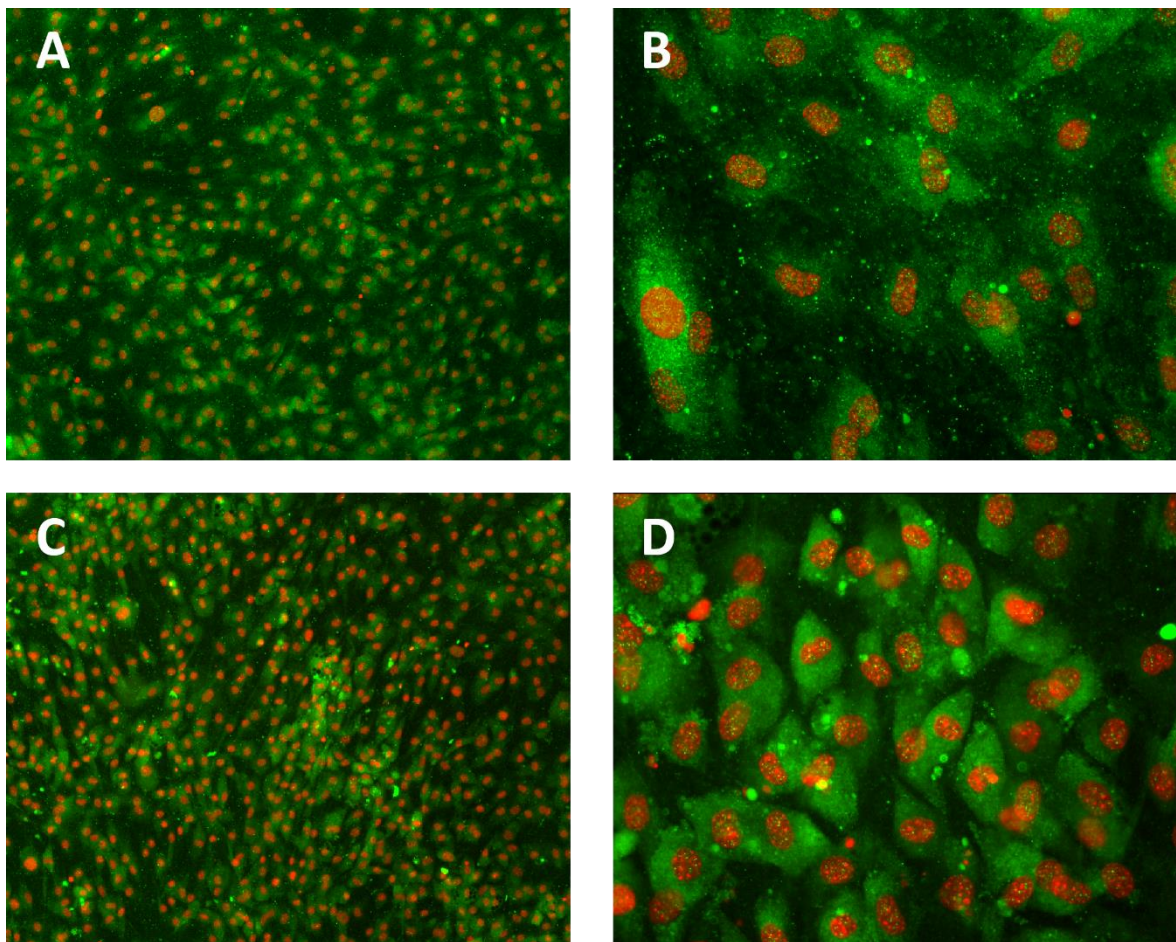


Figure 7. Immunofluorescence of OPN. Cell culture of MSC over crystal slide cover. A and B) Culture with basal medium, at 10X and 40X magnification respectively. C and D) Culture with osteogenic medium, 10X and 40X magnification respectively.

In figure 8, cells cultured over PLLA substrate, with basal medium (A and B) and osteogenic medium (C and D) show similar behavior that those of the cells cultured over C+ (figure 7). Cell density is very similar.

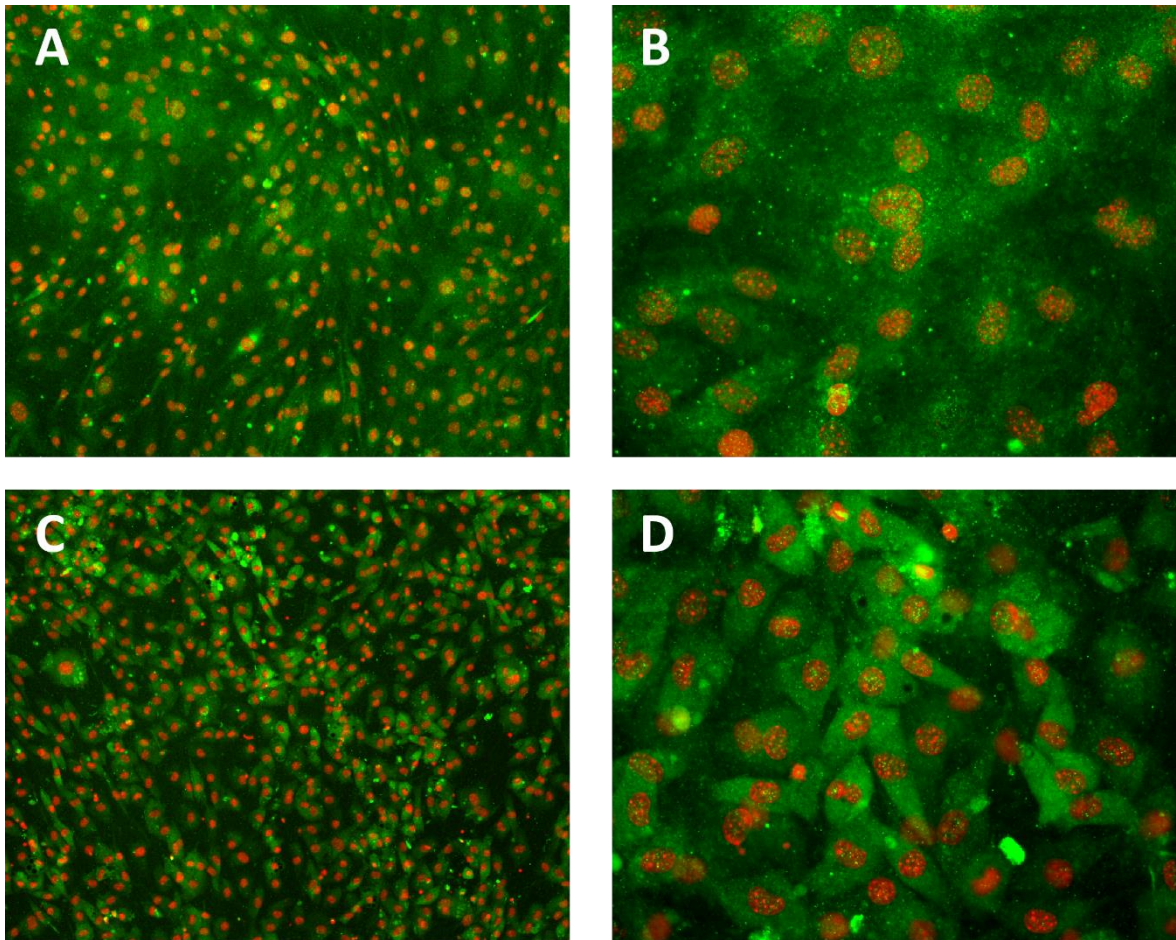


Figure 8. Immunofluorescence of OPN. Cell culture of MSC over PLLA. A and B) Culture with basal medium, at 10X and 40X magnification respectively. C and D) Culture with osteogenic medium, 10X and 40X magnification respectively.

Figure 9 shows the cells cultured over PLLA substrate with boron 2% in the basal (A and B) and osteogenic (C and D) medium. It can be seen the same result as with the other substrates: with osteogenic medium, the cytoplasm is more defined. Cell density seems lower in osteogenic medium, meaning that there are more differentiated cells compared to C+ in osteogenic medium (figure 7C and D).

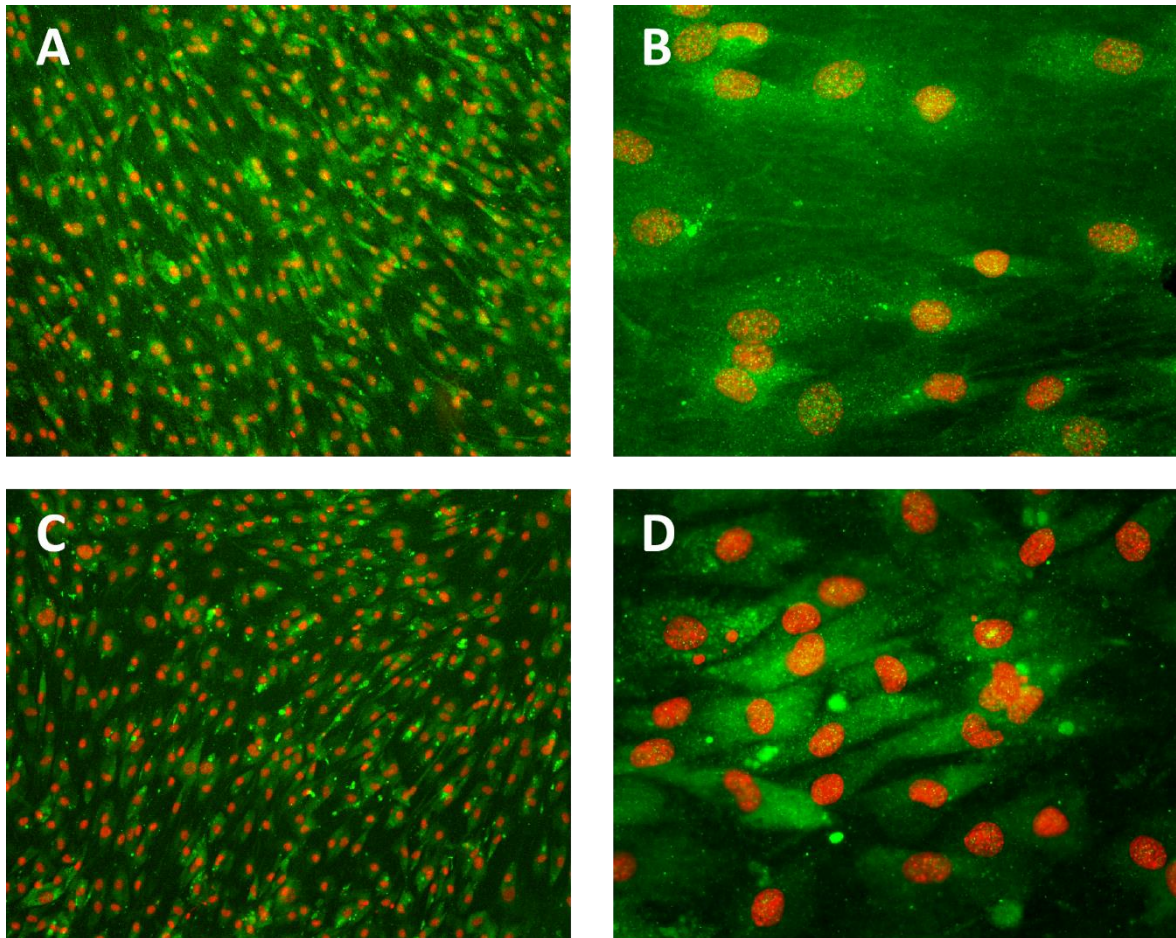


Figure 9. Immunofluorescence of OPN. Cell culture of MSC over PLLA with 2% boron in medium. A and B) Culture with basal medium, at 10X and 40X magnification respectively. C and D) Culture with osteogenic medium, 10X and 40X magnification respectively.

Figure 10 shows the cells cultured over PLLA substrate with boron 5% in the basal (A and B) and osteogenic (C and D) medium. In basal medium, cell density is very low, and cells have an amorphous shape. Cytoplasm is almost no defined. In some cells there is an intense fluorescence next to the nucleus. It is possible that 5% boron concentration is too high for this stage of differentiation and it is being counterproductive, particularly in the case of basal medium, as there is no other osteogenic inductor. In pictures C and D (osteogenic medium), the cytoplasm is more defined than in pictures A and B, but less than the cells with 2% boron. Cell density is low, signaling differentiation.

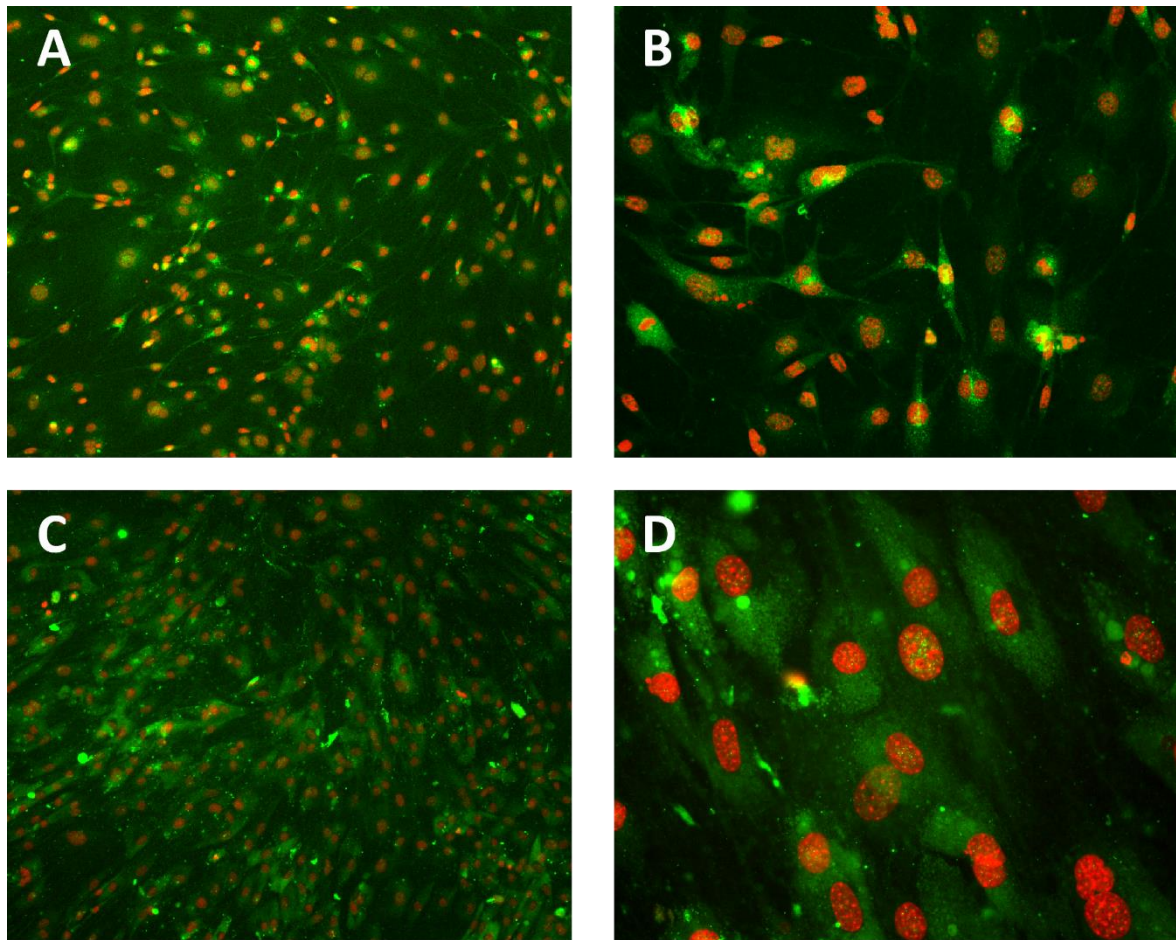


Figure 10. Immunofluorescence of OPN. Cell culture of MSC over PLLA with 5% boron in medium. A and B) Culture with basal medium, at 10X and 40X magnification respectively. C and D) Culture with osteogenic medium, 10X and 40X magnification respectively.

4.3. Myogenesis

The marker chosen for myogenesis was α -actinin sarcomeric. This is an actin-binding protein that is found in muscle. In skeletal muscle it is the major component of z-discs that define muscle sarcomeres, so it is expressed in developed myocytes. DAPI was used to stain the nucleus.

In figure 11 it is shown the culture of MSC over C+, with basal medium (A and B) and myogenic medium (C and D). There is strong background and no definition of cytoplasm in A and B, showing the undifferentiated state of the cells. In myogenic medium (C and D), cells have an elongated shape, characteristic of myocytes and myoblasts. Cell density is much lower, meaning that there are differentiated cells. There are not, however, aligned cells that would indicate the formation of myotubes. It is possible that 15 days are not enough to obtain fully differentiated myoblast cells from mesenchymal stem cells. Rico et al. (2015) obtained myotubes in 4 days from the C2C12 myocyte cell line and Kubo (1991) obtained nucleated skeletal cells in two weeks from

C3H10T1/2 (the same cell line used in this work). However, both used different myogenic medium. It is possible that the chosen medium was not the suitable for this cell line. Another hypothesis is that the obtained cells are myocytes (an early stage of the myogenesis process) and not myoblasts, so α -actinin sarcomeric is less expressed and is not arranged in sarcomeres yet.

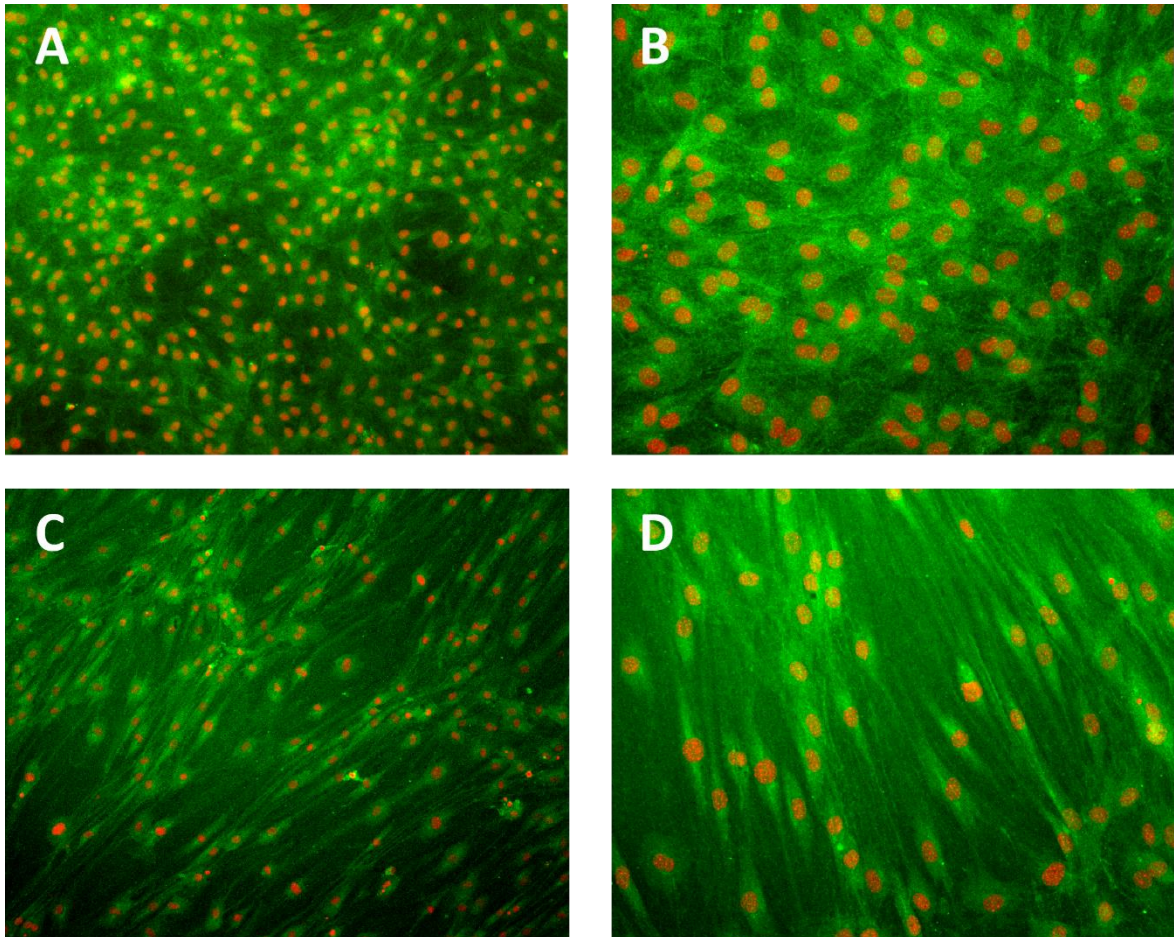


Figure 11. Immunofluorescence of α -actinin sarcomeric. Cell culture of MSC over crystal slide cover. A and B) Culture with basal medium, at 10X and 20X magnification respectively. C and D) Culture with myogenic medium, 10X and 20X magnification respectively.

Figure 12 depicts cells cultured over PLLA substrate, with basal medium (A and B) and myogenic medium (C and D). In basal medium, there is background and high density, same as in figure 11A and B. In C and D pictures, there is lower cell density and cells have lenticular shape, characteristic of myocytes. PLLA is possibly a substrate that enhances myogenesis, compared to slide covers.

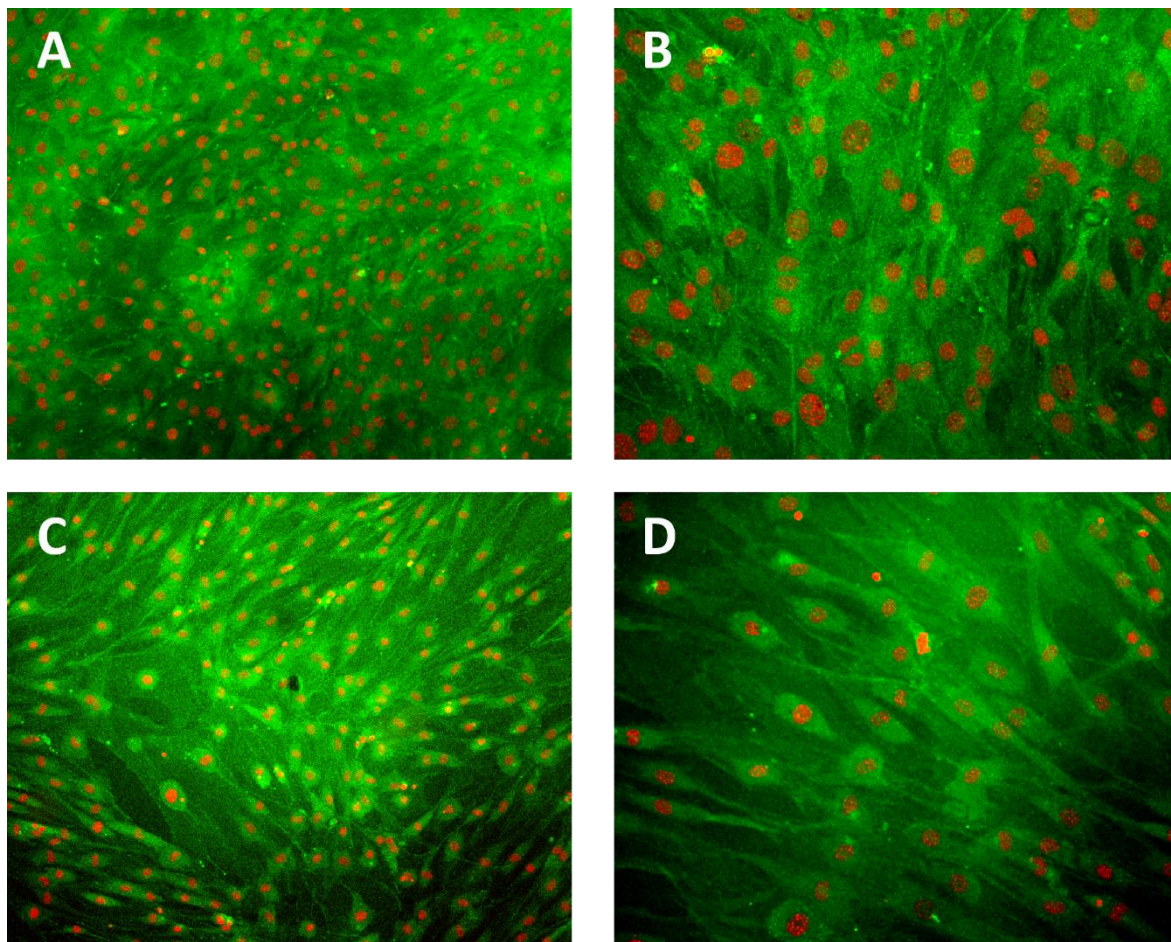


Figure 12. Immunofluorescence of α -actinin sarcomeric. Cell culture of MSC over PLLA. A and B) Culture with basal medium, at 10X and 20X magnification respectively. C and D) Culture with myogenic medium, 10X and 20X magnification respectively.

Figure 13 shows the cells cultured over PLLA substrate with boron 2% in the basal (A and B) and myogenic (C and D) medium. In basal medium there is still high cell density and strong background. However, cells are more defined than in basal PLLA (fig 12A-B) and basal C+ (fig 11A-B) and have an elongated shape. Cells in myogenic medium have the same lenticular shape as depicted in figure 12C-D. Cell density is lower, showing less proliferation and more differentiation.

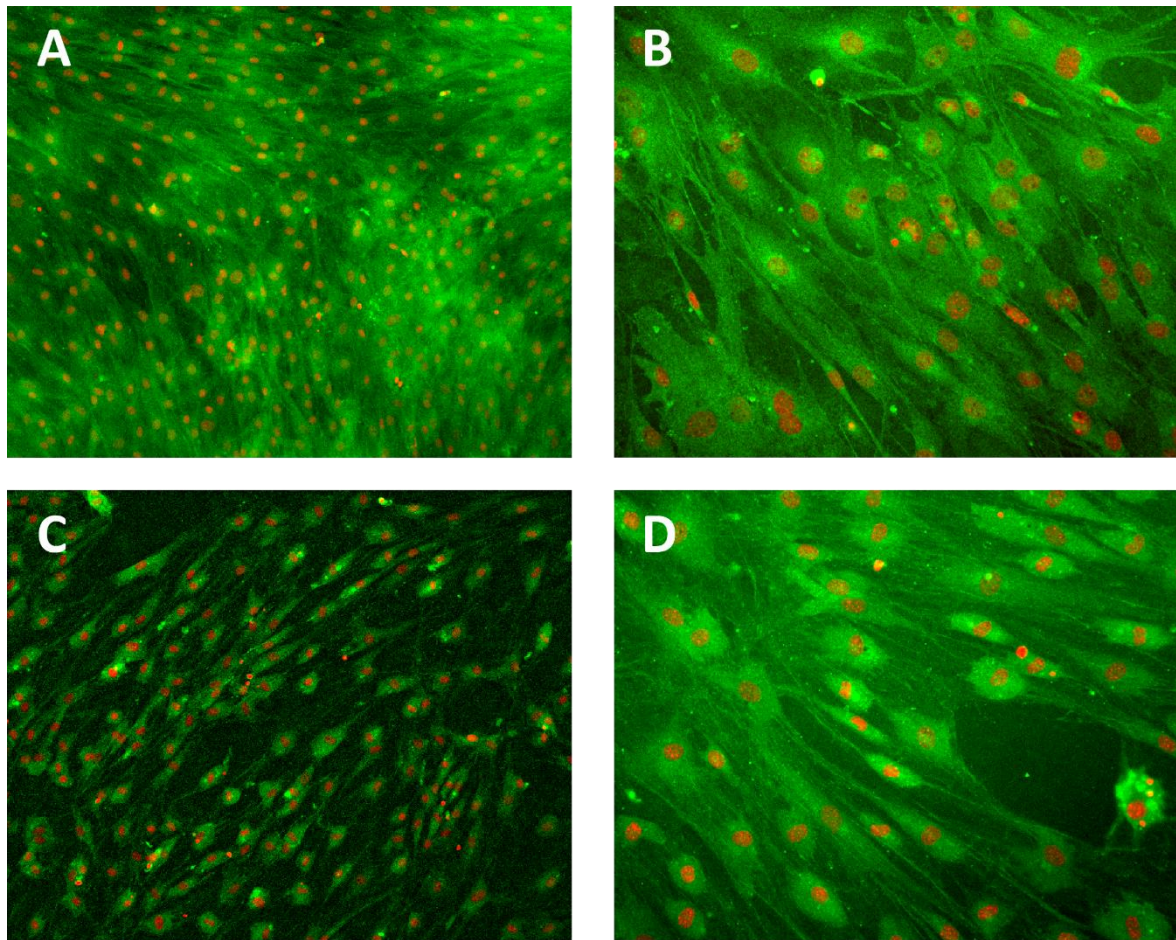


Figure 13. Immunofluorescence of α -actinin sarcomeric. Cell culture of MSC over PLLA with boron 2% in medium. A and B) Culture with basal medium, at 10X and 20X magnification respectively. C and D) Culture with myogenic medium, 10X and 20X magnification respectively.

Figure 14 shows the cells cultured over PLLA substrate with boron 5% in the basal (A and B) and myogenic (C and D) medium. There is less background and less cell density than cultures in boron 2%. Cell cytoplasm is also more defined compared to PLLA and C+ substrates. This means that boron might induce differentiation in basal medium. However, it is not clear the cell morphology and fluorescence is less intense than in the cultures without boron. An explanation can be that spontaneously and without other biochemical factor, boron induces differentiation to another cell line different from myoblast, probably to osteoblast. Pictures C and D show very low cell density, as indicator of differentiation. The morphology of the cell supports the theory that they are myocytes, and 5% of boron improves myogenesis when using myogenic medium.

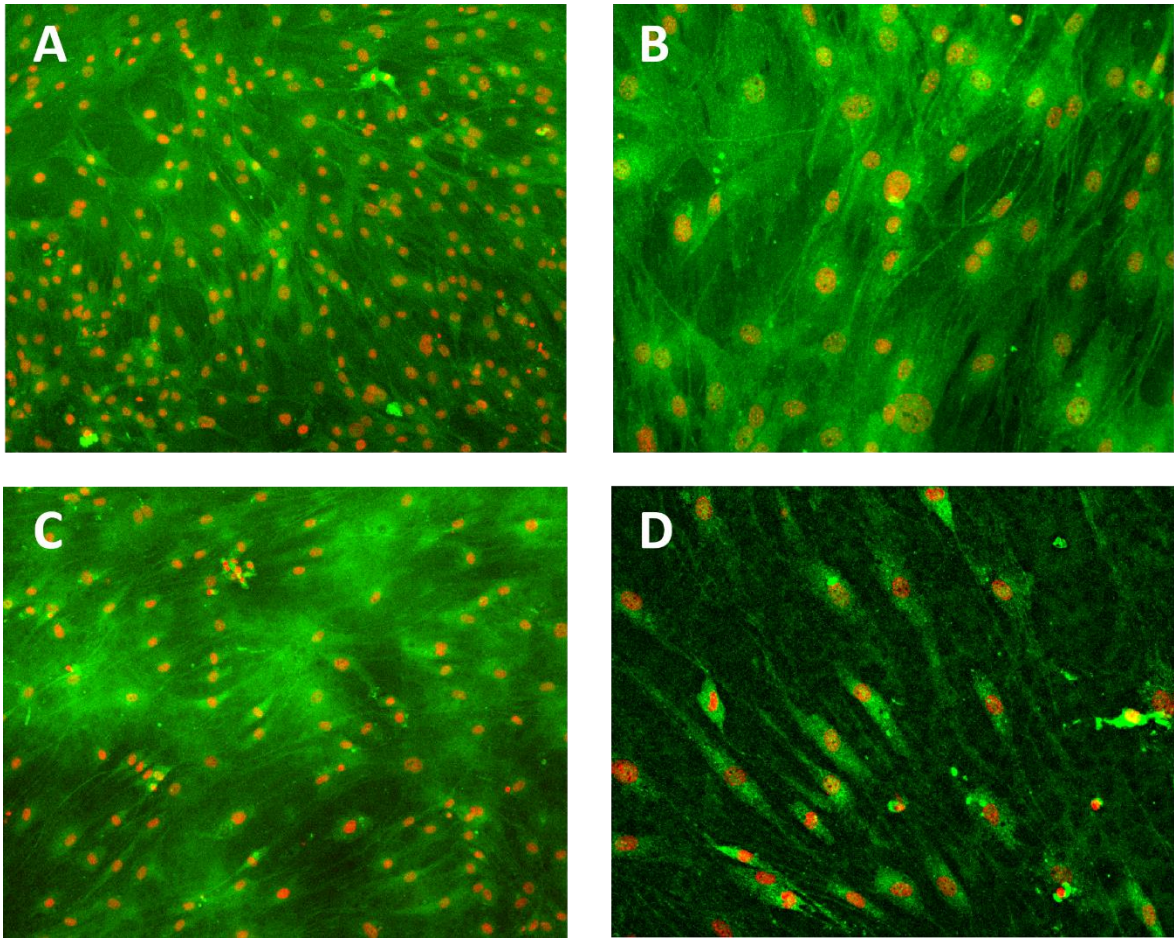


Figure 14. Immunofluorescence of α -actinin sarcomeric. Cell culture of MSC over PLLA with boron 5% in medium. A and B) Culture with basal medium, at 10X and 20X magnification respectively. C and D) Culture with myogenic medium, 10X and 20X magnification respectively.

4.4. Adipogenesis

To test for adipogenic differentiation, an Oil Red O staining was performed. Oil Red O is a fat soluble dye which stains triglycerides and lipids in red. It makes visible the fat deposits in adipocytes.

In figure 15 it is shown the culture of MSC over C+, with basal medium (A and B) and adipogenic medium (C and D). No red staining can be seen in basal medium, meaning there is not any adipocyte. By contrast, in adipogenic medium the stained fat vacuoles can be easily located. Cell density in the second case is low.

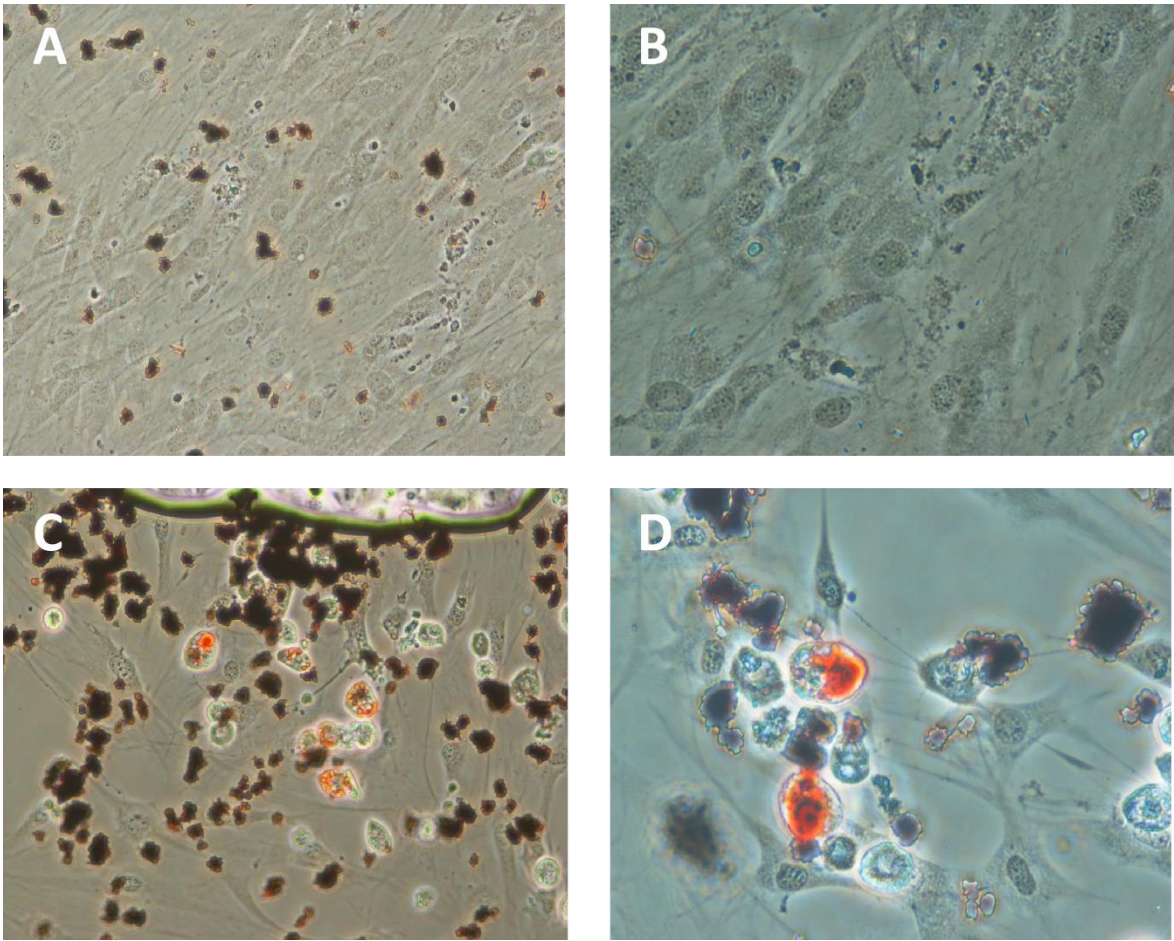


Figure 15. Staining of adipocytes. Cell culture of MSC over crystal slide cover. A and B) Culture with basal medium, at 20X and 40X magnification respectively. C and D) Culture with adipogenic medium, 20X and 40X magnification respectively.

Figure 16 shows cells cultured over PLLA substrate, with basal medium (A and B) and adipogenic medium (C and D). Basal medium has no staining. In adipogenic medium, the amount of adipocytes is much higher than in C+. Possibly PLLA enhances adipogenesis.

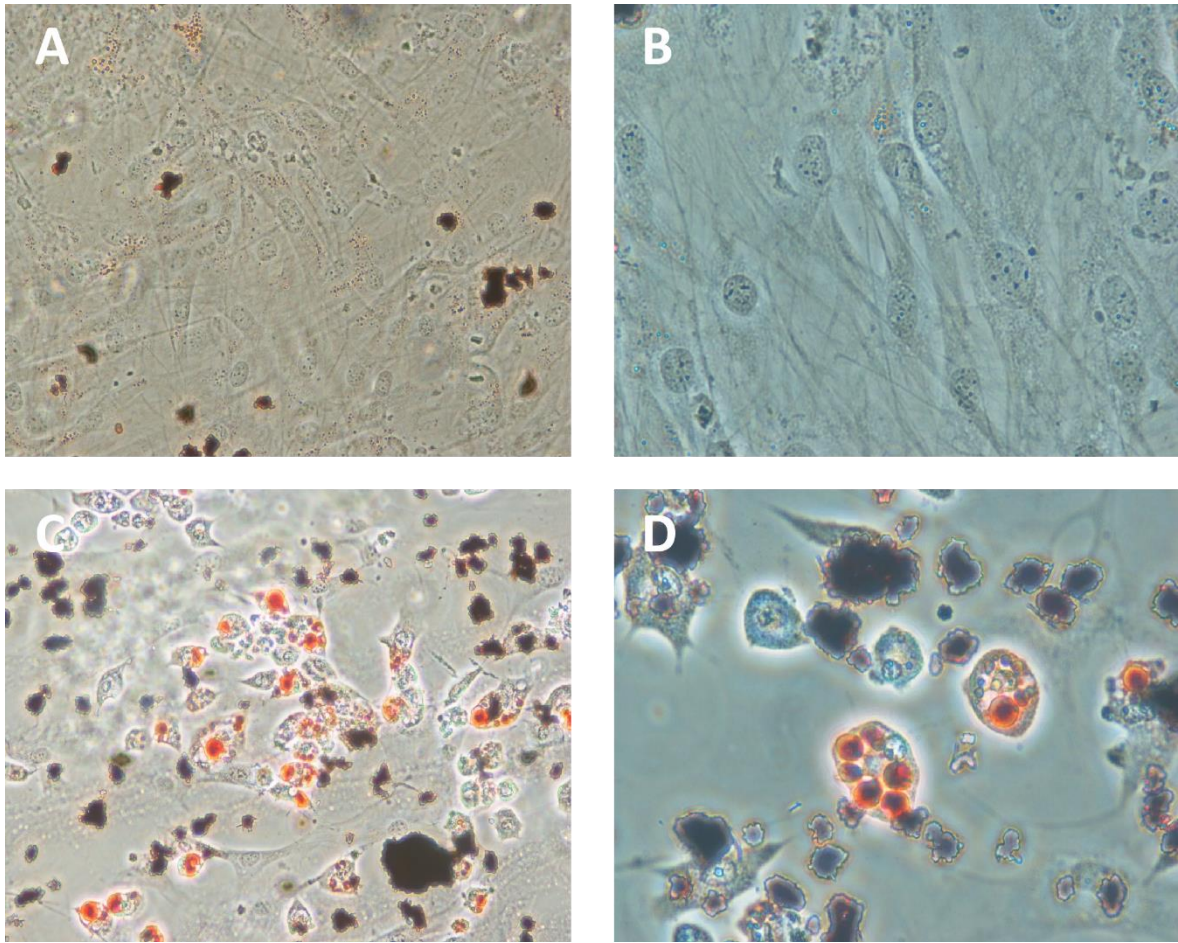


Figure 16. Staining of adipocytes. Cell culture of MSC over PLLA. A and B) Culture with basal medium, at 20X and 40X magnification respectively. C and D) Culture with adipogenic medium, 20X and 40X magnification respectively.

Figure 17 shows the cells cultured over PLLA substrate with boron 2% in the basal (A and B) and adipogenic (C and D) medium. In the basal medium, the cell density is slightly lower than in PLLA and C+ substrates without boron. There are no stained cells. In adipogenic medium, there are fewer adipocytes than in both PLLA and C+. Additionally, adipocyte morphology is atypical - not rounded. They have a few ramifications and show a polygonal shape. Cell density is very low, depicting differentiation.

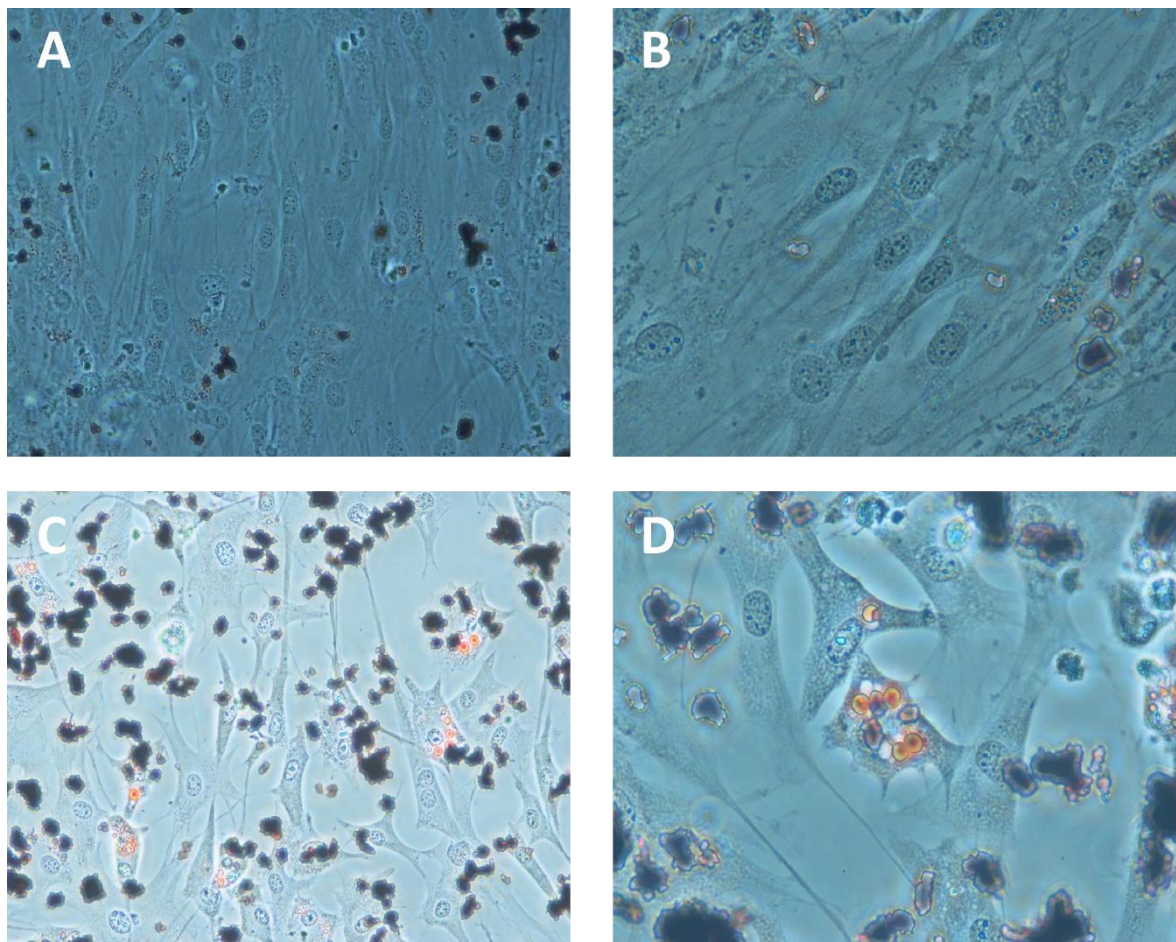


Figure 17. Staining of adipocytes. Cell culture of MSC over PLLA with 2% boron in medium. A and B) Culture with basal medium, at 20X and 40X magnification respectively. C and D) Culture with adipogenic medium, 20X and 40X magnification respectively.

Figure 18 shows the cells cultured over PLLA substrate with boron 5% in the basal (A and B) and adipogenic (C and D) medium. In A and B cell density is much lower compared to the other conditions with basal medium. Cell morphology is different from fibroblast, so possibly they are differentiated cells. Without the proper marker it can't be confirmed, but the branched shape indicates that they can be pre-osteoblasts. C and D figures show even less adipocytes than PLLA+B2% (figure 17C-D). The few adipocytes have again a branched and polygonal shape, typical of pre-osteoblasts in culture.

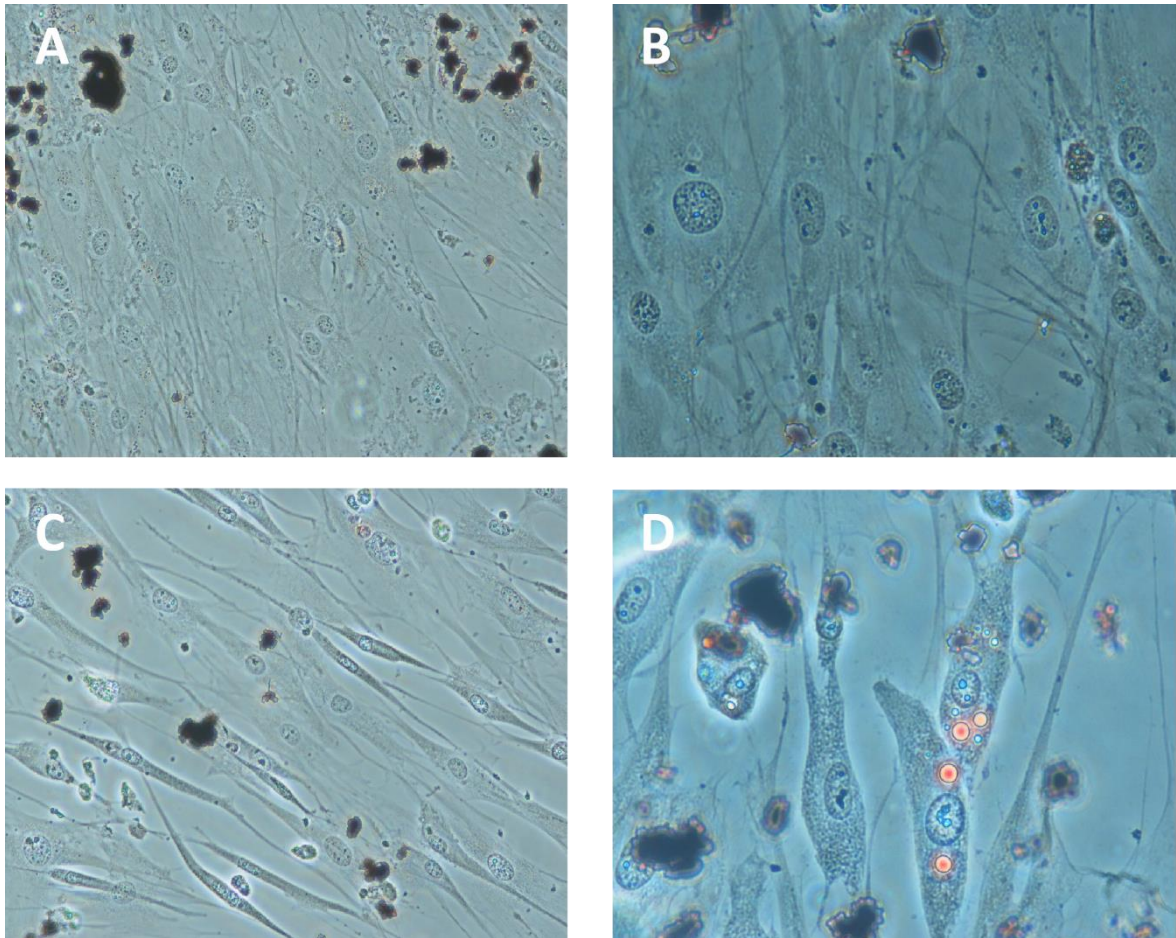


Figure 18. Staining of adipocytes. Cell culture of MSC over PLLA with 2% boron in medium. A and B) Culture with basal medium, at 20X and 40X magnification respectively. C and D) Culture with adipogenic medium, 20X and 40X magnification respectively.

This provides more support to the hypothesis that boron inhibits the adipogenesis pathway by favoring osteogenesis. It is known that RunX2 blocks adipogenesis (Komori 2010). If boron activates RunX2, then it is consequent that in the presence of boron, adipogenesis is blocked. However, more experiments that lead to quantitative results are required.

5. Conclusions

The objective of this research was to test the effect of boron on the differentiation of mesenchymal stem cells. The hypothesis, based on literature review, was that boron very probably induces osteogenesis, and possibly induces myogenesis. The mechanisms are unclear and it is not known at what stage of differentiation boron acts.

The results shown here provide more insight on how boron induces cell differentiation. The analysis of the immunofluorescence assays of RunX2 suggest that boron acts at an early stage of differentiation, at 2% and 5% boron in culture medium. The morphology of the cells and the fluorescence in osteogenic medium in the positive control (slide cover) denote that 3-day time to induce osteogenesis is too short time. Even so, boron 5% was able to induce the expression of RunX2 and hence begin the process of osteogenesis. When analyzing OPN immunofluorescence, it points out that boron 2% induces differentiation of osteoblasts, as the results are better than the positive control. However, in the culture with basal medium + boron 5% the cells do not produce OPN, and in osteogenic medium + boron 5% the grade of differentiation seems lower than that in positive control. This can mean that at 15-day, at later stages of osteogenesis, that concentration of boron is counterproductive for the osteoblast, as OPN is not produced in later immature osteoblasts.

As negative control, the adipogenesis experiment showed how increasing concentrations of boron not only reduced adipogenesis, but also seemed to induce osteogenesis. If boron activates RunX2, then it is expected that adipogenesis is blocked, as the transcription factor avoids the expression of adipogenic inducer genes. Additionally, in basal medium, without any biochemical inducer, there can be seen cells with a pre-osteoblast morphology. Therefore, it can be concluded that boron induces osteogenesis, probably in early stages of the process, and probably activating RunX2, which is one of the first transcription factors to be activated in the differentiation of MSC. More experiments are needed to confirm this hypothesis. A quantitative experiment measuring level of expression of particular genes could be the most suitable choice.

Myogenesis experiments are less concluding. The most plausible explanation is that the myogenic media was not correct for the used cell line, making the differentiation process slower than expected. Being the myogenesis slower is logical that cells differentiated to myocytes and not myoblasts (a more advanced stage). The marker chosen, α -actinin sarcomeric, gives the characteristic aligned shape of myotubes only when cells are fused myoblasts. As this was not the

case, the marker did not allow seeing the proper morphology of the cell, making difficult the analysis of the differentiation. Nevertheless, the lower cell density and the lenticular shape in B2% and B5% myogenic media show that to a certain extent boron might enhance myogenesis, but possibly in a more advanced stage of differentiation. More experiments need to be done to confirm this.

It is interesting to notice the morphology of the cells and the cell density in B2% and B5% basal media of myogenesis experiment. Even if it is not a differentiation media, cells are differentiated to some lineage. As the marker chosen is for myogenesis, it can't be certain what the lineage is. The same phenomena can be slightly observed in the Sca1 experiment: cells seem differentiated due to their shape in the culture. However, as the marker is for stemness, it is not clear which cell type might be. One hypothesis is that those cells are pre-osteoblasts, because boron shown in the adipogenesis and osteogenesis experiment that it can induce osteogenesis without any additional biochemical factor. To test this, it would be necessary to use other proper markers.

The next step for this research is to confirm these results and test the hypothesis that **boron induces osteogenesis without any additional factor and enhances myogenesis under particular conditions**. The most reliable way to do this is to make a quantitative analysis of mRNA expression in the cells, to test the markers that represent various stages of differentiation of both lineages.

The knowledge acquired in how boron acts and at what levels, makes tissue engineering to move one step forward, towards the obtaining of a new biochemical factor that will help in bone and muscle regeneration. As something to notice, PLLA enhanced in all cases cell differentiation, compared to slide covers. This reminds that the synergy between the biomaterial, the biochemical factor and the cells must be kept in order to improve tissue engineering as a whole, taking advantage of all of its sides to make every day this fascinating discipline a real application for biomedicine in the real world.

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