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SILICON AND PLATELET CONCENTRATES IN TISSUE REGENERATION: AN "IN VITRO" STUDY.

Coordinator:
Professor CHIARELLA SFORZA

Tutor:
Professor LUIGI FABRIZIO RODELLA

PhD student:
VERONICA BONAZZA

Registration number: R10439

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To my family.....

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Abstract

Tissue regeneration is a complex process of healing and tissue growth, which involves different biological elements and strategies, including the use of autologous cells, growth factors and scaffolds. All of these elements are present in platelet concentrates, such as Concentrated Growth Factors (CGF). Moreover, some trace elements play an important role in enhancing cell growth and proliferation and so tissue development and regeneration. Among these, Silicon seems to be beneficial for different organs and tissues, such as: bone and cartilage, brain, skin, nails and hair, cardiovascular system and immune system. For this reason, in the present study we evaluated the *in vitro* effect of Silicon (in the soluble form of Sodium Orthosilicate) and CGF on three different human cell lines of fibroblasts (NHDF), endothelial cells (HUVEC) and osteoblasts (HOBs). Each cell type, was treated with Sodium Orthosilicate at the final concentration of 0,5 mM and 1 mM, CGF and CGF supplemented with Sodium Orthosilicate, for 72 hours. At the end of the experimental period, the effect of the different treatments, on cell growth, proliferation and metabolic activity was evaluated by performing a simple cell count, using an automated cell counter and by evaluating the expression of the intracellular proliferation marker Ki-67, using FACS. Moreover, the expression of other cell markers and active molecules such as Collagen type I (Col I), Osteopontin (OPN), Vascular Endothelial Growth Factor (VEGF) and endothelial Nitric Oxide synthase (eNOS), was evaluated, through immunohistochemical analyses on fixed cells. Results obtained showed that the use of CGF in combination with Sodium Orthosilicate stimulates cell growth, proliferation and metabolic activity. Overall, these findings suggest that *in vitro* treatment with CGF and Sodium Orthosilicate seems to be promised in promoting cell growth and proliferation and so in tissue regeneration.

1 INTRODUCTION

1.1 Tissue regeneration

Regeneration is a regulative developmental process, ubiquitous across all species. It functions throughout the life cycle to maintain or restore the normal form and function of cells, tissues and in some cases organs, appendages and whole organisms. In fact the term “regeneration” refers to a type of healing in which new growth completely restores damaged tissue or lost appendage, to their normal state (*Forbes et al., 2014; Krafts et al., 2010*). Regeneration requires dramatic changes in cellular behaviour and includes different processes such as wound healing, cell death, dedifferentiation and stem cell proliferation. Regeneration can be achieved by restoration, intended as putting together what is broken and by reconstruction, defined as the replace and rebuilt of what it is torn down. On the contrary, the term repair, is used to refer to the reestablishment of tissue continuity, through restoration of tissue architecture and function. While a few types of tissue injury can sometimes be healed so that no permanent damage remains, most of the body’s tissue repair involves both regeneration and replacement. These pathways depend, in part, on the type of tissue in which occur. In fact, certain tissues of the body are more capable of cellular proliferation and so regeneration, than others. In this regard, we can distinguish three types of tissues: 1) continuously dividing tissues, 2) quiescent tissues and 3) non dividing tissues (*Krafts et al., 2010*). Continuously dividing tissues (or labile tissues), are characterized by cells in constant proliferation. Examples of these tissues include epithelia (such as skin, gastrointestinal epithelium and salivary gland tissue) and hematopoietic tissues. These tissues contain pools of stem cells, which have enormous proliferative and self-renewing ability and which give rise to more than one type of cell. Quiescent tissues (or stable tissues) are composed of

cells that normally exist in a non-dividing state but may enter in the cell cycle in response to certain stimuli, such as cell injury. Examples of these tissues include parenchymal cells of the liver, kidney and pancreas, mesenchymal cells such as fibroblasts and smooth muscle cells, endothelial cells and lymphocytes. Non dividing tissues (or permanent tissues) are composed of cells that have left the cell cycle permanently and are therefore unable to proliferate. Examples of these tissues include cardiac and skeletal muscle. Tissue repair in these tissues always leaves permanent evidence of injury, such as a scar.

1.2 Key elements in tissue regeneration

Tissue regeneration is a complex process of healing and tissue growth, which involves different biological elements and strategies. These include the use of bone grafts (*García-Gareta et al., 2015*), biomaterials and growth factors, natural or synthetic scaffolds (*Asti et al., 2014; Loh et al., 2013)* and the use of autologous cells (stem cells), (Figure 1) (*Manunta et al., 2016*; *Fan et al., 2016; Polak et al., 2006)*.

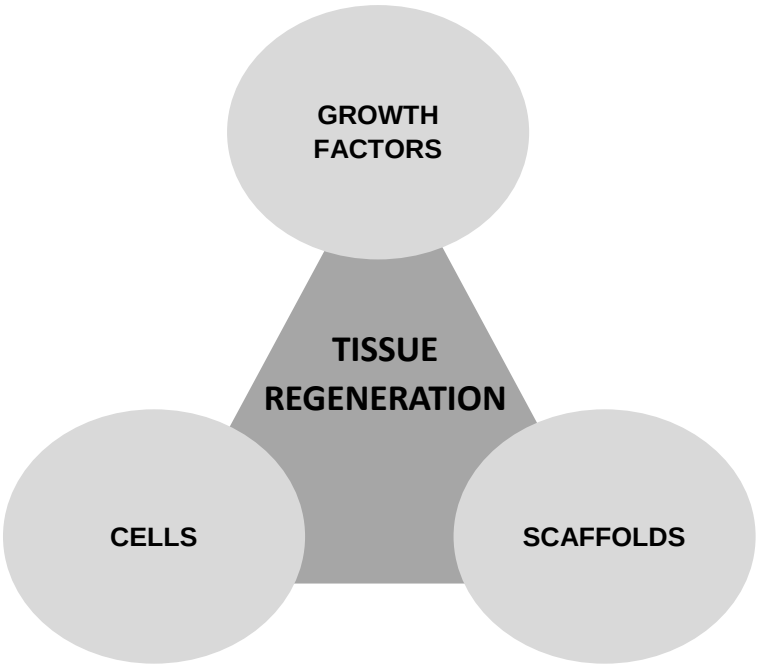

Figure 1: Key elements in tissue regeneration

1.2.1 Cells in tissue regeneration

1.2.1.1 Stem cells

Stem cells are the focus of many applications in regenerative medicine because of their extensive ability to self-renew and to generate differentiated progeny (*Watt et al., 2010*). Stem cells are the foundation for every organ and tissue in our body. They are undifferentiated cells, which differ from other kinds of cells of the body, having three general properties: 1) they are able to divide and renew (make copies of themselves) for long periods 2) they are unspecialized and 3) they can differentiate, giving rise to more specialized cells. For this reason these cells are used to enhance the regenerative capacity of different tissues and organs and for the treatment of a wide variety of diseases, such as neurological disorders (*Donegan et al., 2016; Ferreri et al., 2016*), autoimmune and other immunological disorders (*Yarygin et al., 2016*), cardiovascular diseases (*Faiella et al., 2016),* bone and cartilage diseases *(Burke et al., 2016; Li et al., 2015)* and many other diseases.

There are many different types of stem cells that come from different places in the body or are formed at different times in our life. In particular, we can divide stem cells into four categories:

- Embryonic stem cells (ESCs)

- Adult stem cells (ASCs)

- Mesenchymal stem cells (MSCs)

- Induced pluripotent stem cells (iPSCs)

1.2.1.2 Embryonic stem cells (ESCs)

Human embryonic stem cells (ESCs) have been derived primarily from the inner cell mass of the blastocyst (*Reubinoff et al., 2000; Thomson et al., 1998,1995*), a mainly hollow ball of cells that, in the human, is formed three to five days after an egg cell is fertilized by a sperm (Figure 2).

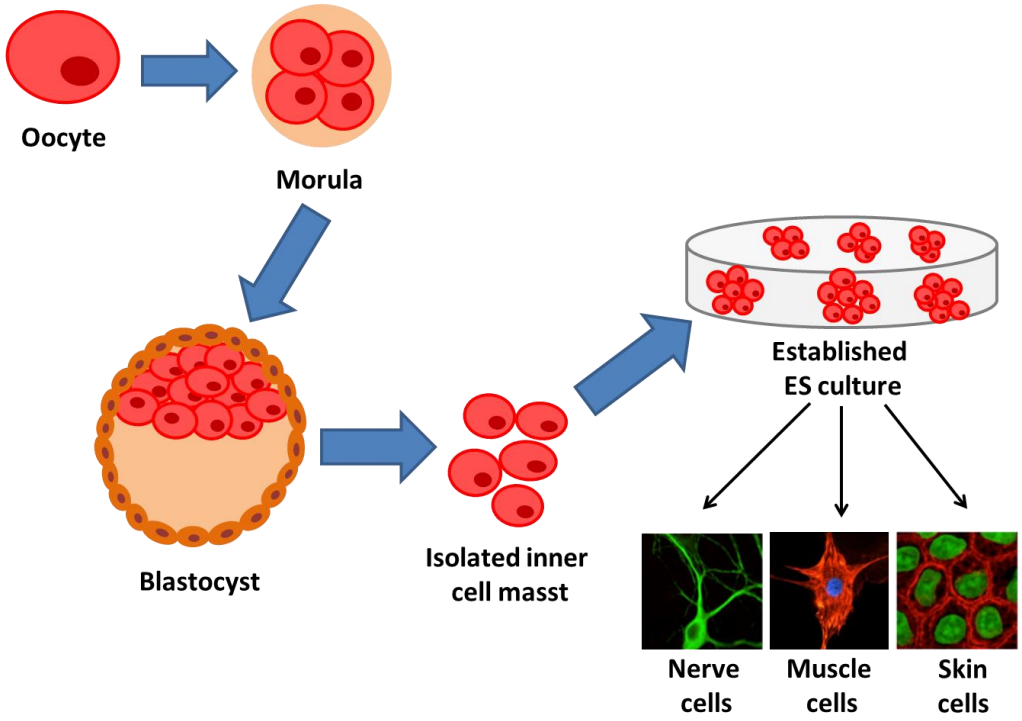

Figure 2: Establishment of ES cell lines: the inner cell mass of the blastocyst contains pluripotent cells that are isolated and propagated in tissue culture. ES cells can proliferate indefinitely and maintain their pluripotent state.

Embryonic stem cells are pluripotent cells, so they are able to differentiate into a myriad of cell types and have the ability of "self-renewal", meaning that unlimited numbers of identical, well-defined, genetically and genomically characterized stem cells can be produced in culture for medical use. These cells are incredibly valuable

because they provide a renewable resource for studying normal development and disease and for testing drugs and other therapies.

1.2.1.3 Ault stem cells (ASCs)

Adult stem cells (ASCs) are more specialized than embryonic stem cells (*Young et al., 2004*). Typically, these stem cells can generate different cell types for the specific tissue or organ in which they live. Unlike ESCs, which are defined by their origin (the inner cell mass of the blastocyst), ASCs share no such definitive means of characterization and they don't seem to self-renew in culture as easily as embryonic stem cells do. The list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver and pancreas. In order to be classified as an adult stem cell, the cell should be capable of self-renewal for the lifetime of the organism and it should be clonogenic, meaning that a single adult stem cell should be able to generate a line of genetically identical cells, which then gives rise to all the appropriate differentiated cell types of the tissue in which it resides. An adult stem cell should also be able to give rise to fully differentiated cells that have mature phenotypes, are fully integrated into the tissue and are capable of specialized functions that are proper for the tissue. There are different types of adult stem cells such as: hematopoietic stem cells (*Seita et al., 2010*), mammary stem cells (*Soady et al., 2015*; *Visvader et al., 2014; Liu et al., 2005*), intestinal stem cells (*Van der Flier et al., 2009; Barker et al,. 2008*), endothelial stem cells (*Kim et al., 2008*), neural stem cells (*Gage et al., 2013; Schmittwolf et al., 2005*), olfactory stem cells (*Féron et al., 2013*; *Murrell et al., 2005)* testicular stem cells (*Conrad et al., 2008; Hamra et al., 2008; Goossens et al., 2006*).

1.2.1.4 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are defined as self-renewing and multipotent cells capable of differentiating into multiple cell types, including osteocytes, chondrocytes, adipocytes, hepatocytes, myocytes, neurons and cardiomyocytes (*Bobis et al., 2006*), (Figure 3).

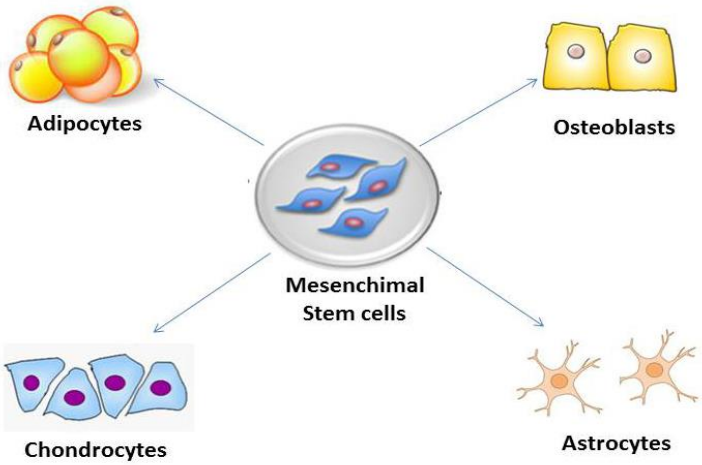

Figure 3: MSCs are multipotent cells capable of differentiating into different cell types.

MSCs were originally isolated from the bone marrow stroma but they have recently been identified also in other tissues, such as adipose tissue, epidermis, peripheral blood, cord blood, liver and fetal tissues (*Gnecchi et al., 2009; Beyer et al., 2006*). Different methods have been used for MSCs isolation. Among these, the most common is based on the ability of the MSCs to selectively adhere to plastic surfaces. MSCs play a key role in the maintenance of bone marrow homeostasis and in the regulation of both hematopoietic and non-hematopoietic cells maturation. These cells represent a powerful tool in gene therapies and can be effectively transduced with viral vectors containing a therapeutic gene, as well as with cDNA for specific proteins. MSCs are attractive for clinical therapy due to their ability to differentiate, provide trophic support and modulate innate immune response (*Phinney et al., 2007*). They

have been used in bone and cartilage repair (*Shao et al., 2015; Wang et al., 2013; Undale et al., 2009*), heart and blood vessels repair (*White et al., 2016; Karantalis et al., 2015; Kim et al., 2015*), inflammatory and autoimmune diseases (*Klinker et al., 2015; Newman et al., 2009*).

1.2.1.5 Induced pluripotent stem cells (iPSCs)

A relatively recent breakthrough in stem cell research is the discovery that specialized adult cells can be 'reprogrammed' into cells that behave like embryonic stem cells, named as induced pluripotent stem cells (iPSCs). The pioneer of the iPSC technology in mouse, was Shinya Yamanaka and his team at Kyoto University in 2006, showing that the introduction of four specific genes encoding transcription factors could convert adult cells into pluripotent stem cells (*Takahashi et al., 2006*). Human iPSCs were first reported one year later, in 2007. iPSCs are obtained, by introducing a specific set of reprogramming factors, into a given cell type. These reprogramming factors (also dubbed Yamanaka factors) include the transcription factors Oct4 (Pou5f1), Sox2, cMyc, and Klf4. iPSC's production is a slow and inefficient process, taking 1–2 weeks for mouse cells and 3–4 weeks for human cells, with efficiencies around 0.01%–0.1%. Even if iPSCs share many of the same characteristics of embryonic stem cells, including the ability to give rise to all the cell types in the body, they aren't exactly the same. The discovery of iPSCs represents an important tool to help scientists to learn more about normal development and disease onset and progression, and also to develop and test new drugs and therapies. For example, researchers have generated brain cells from iPSCs made from skin samples belonging to patients with neurological disorders such as Down's syndrome or Parkinson's disease (*Du et al., 2015; Russo et al., 2015*). These lab-

grown brain cells show signs of the patients' diseases. This has implications for understanding how the diseases actually happen and for searching and testing new drugs. However the technology is very new and the reprogramming process is not yet well understood.

1.2.1.6 Blood cells as a source of growth factors

Among blood cells, platelets represent an important source of growth factors. Platelets are produced by megakaryocytes as anucleated cells. A variety of growth factors, coagulation factors, adhesion molecules, cytokines, chemokines and integrins are stored in platelets and in particular in their alfa granules. After activation, platelets release a multitude of growth factors at concentrations significantly higher than the baseline blood levels, including transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and many others. Growth factors released from activated platelets initiate and modulate wound healing and regeneration in both soft and hard tissues.

1.3 Growth factors

Growth factors (GFs), are soluble-secreted signaling polypeptides that activate and guide different cellular processes involved in tissue healing, such as infiltration, growth, differentiation, migration, cell metabolism and apoptosis (*Lee et al., 2011*). They modulate cellular activity by acting as signaling molecules between cells, transferring information between cell populations and their micro-environment, resulting in accelerated functional reparation of the damaged tissues. GFs

accomplish their function binding to specific transmembrane receptors on the surface of target cells. The instructions are translated into the cell through complex signal transduction networks resulting in a specific biologic cellular response. Common growth factors involved in tissue regeneration include: Transforming Growth Factor Beta 1 (TGF-β1), Bone Morphogenetic Proteins (BMPs), Insulin-like Growth Factor 1 (IGF-1), Vascular endothelial Growth Factor (VEGF), Tumor Necrosis Factor alpha (TNF-α), Fibroblast Growth Factor (FGF) and Platelet Derived Growth Factor (PDGF).

1.3.1 Transforming Growth Factor Beta 1 (TGF-β1)

TGF-β1 is a polypeptide member of the TGF-β superfamily of cytokines. It is a growth factor ubiquitously expressed and it was initially discovered as a factor inducing colony formation of normal rat kidney fibroblasts in soft agar in the presence of epidermal growth factor (EGF) By immunohistochemical techniques TGF-β1 was strongly detected in adrenal cortex, megakaryocytes and other bone marrow cells, cardiac myocytes, chondrocytes, renal distal tubules, ovarian glandular cells and chorionic cells of the placenta and also in cartilage, heart, pancreas, skin and uterus. TGF-β1 is secreted as an inactive precursor bound to the Latency Associated Peptide (LAP), forming the complex called Small Latent Complex (SLC). SLCs are secreted from cells and deposited into the extracellular matrix as covalent complexes with its binding proteins, also known as Latent TGF-β Binding Proteins, LTBPs (*Koli et al., 2001*). The latency proteins contribute to TGF-β1 stability. Active TGF-β1 half-life is about two minutes whereas LTBPs half-life is about 90 minutes. In cells, active TGF-β1 is forming a large ligand-receptor complex involving a ligand dimer and four receptor molecules. TGF-β1 performs many cellular functions, including the control of

cell growth, cell proliferation, cell differentiation and apoptosis. It plays an important role in embryonic development, tissue morphogenesis, cell proliferation and differentiation (*Ramoshebi et al., 2002*) and also in bone induction, remodeling and extra cellular matrix (ECM) production (*Urist et al., 2002*).

1.3.2 Bone Morphogenetic Proteins (BMPs)

BMPs are multi-functional growth factors that belong to the TGF beta superfamily (*Ducy et al., 2000*). Originally discovered by their ability to induce the formation of bone and cartilage, BMPs are now considered to constitute a group of essential morphogenetic signals, orchestrating tissue architecture throughout the body. Originally, seven such proteins were discovered: six belonging to the TGF beta superfamily and one (BMP1) is a metalloprotease. Since then, thirteen more BMPs have been discovered, bringing the total to twenty. Among these proteins, BMP-2 and BMP-7, are involved in the formation of bone and cartilage and they represent the most extensively studied proteins to induce bone formation even in critical size defects (*Choi et al., 2016; Kolk et al., 2016; Betz et al., 2015; Del Rosario et al., 2015*). Recombinant human BMPs (rhBMP-2 and rhBMP-7) are Food and Drug Administration (FDA)-approved and they are used in orthopedic applications such as spinal fusions (*Liao et al., 2003*), non-unions and oral surgery (*Boyne et al., 2005*).

1.3.3 Insulin-like Growth Factor 1 (IGF-1)

Insulin-like growth factor 1 (IGF-1), also called somatomedin C, is a protein that in humans is encoded by the IGF-1 gene. It has a molecular structure similar to insulin. IGF-1 is produced primarily by the liver as an endocrine hormone as well as in target

tissues in a paracrine/autocrine manner. It is produced throughout life and the highest rates of IGF-1 production occur during the pubertal growth spurt, whereas the lowest levels occur in infancy and old age. Its primary action is mediated by binding to its specific receptor, the insulin-like growth factor 1 receptor (IGF-1R), which is present on many cell types in many tissues. In this way, IGF-1 initiates intracellular signalling. In fact it is one of the most potent natural activators of the AKT signaling pathway, a stimulator of cell growth and proliferation, and a potent inhibitor of programmed cell death. IGF-1 is involved in the regulation of neural development including neurogenesis, myelination, synaptogenesis, dendritic branching and neuroprotection after neuronal damage. Moreover this growth factor represents a critical mediator of bone growth and fracture healing, promoting cell proliferation and matrix synthesis by chondrocytes and osteoblasts (*Locatelli et al., 2014*).

1.3.4 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF), originally known as vascular permeability factor (VPF), is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. One of the main functions of VEGF is to form new blood vessels during the growth and to stimulate the growth of new blood vessels after injury. In cases where blood vessels are obstructed, VEGF also promotes the creation of new blood vessels to bypass the blocked vessels.

1.3.5 Tumor Necrosis Factor alpha (TNF-α)

TNF-α is a cell signaling protein (cytokine) involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. TNF was thought to be

produced primarily by macrophages, but it is produced also by a wide variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, eosinophils and neurons. TNF-α can bind two receptors, TNFR1 and TNFR2. It exerts many effects on different organ systems, generally together with Interleukin 1 (IL-1) and 6 (IL-6): on the hypothalamus it stimulates the release of corticotropin releasing hormone (CRH), on the liver it stimulates the acute phase response, leading to an increase in C-reactive protein and a number of other mediators. It also induces insulin resistance by promoting serine-phosphorylation of insulin receptor substrate-1 (IRS-1), which impairs insulin signaling. Moreover it is a potent chemoattractant for neutrophils and promotes the expression of adhesion molecules on endothelial cells, helping neutrophils to migrate. On macrophages, TNFα stimulates phagocytosis and production of IL-1 oxidants and the inflammatory lipid Prostaglandin E2 (PGE2).

1.3.6 Fibroblast Growth Factor (FGF)

Fibroblast growth factors (FGFs) are secreted molecules which function through the activation of specific tyrosine kinases receptors, the FGF receptors, that transduce the signal by activating different pathways including the Ras/MAP kinase and the phospholipase-C gamma pathways. FGFs are involved in angiogenesis, wound healing, embryonic development and various endocrine signalling pathways. During the development of the central nervous system, FGFs play important roles in neural stem cell proliferation, neurogenesis, axon growth and differentiation. In humans, 22 members of the FGF family have been identified. They are key players in the processes of proliferation and differentiation of wide variety of cells and tissues.

1.3.7 Platelet Derived Growth Factor (PDGF)

PDGF is one of the numerous growth factors or proteins that regulate cell growth and division. In particular, it plays an important role in angiogenesis, promoting the formation and growth of new blood vessels and regulating vascular tone and platelet aggregation. PDGF plays a central role also in cardiovascular diseases, as for example during vessel remodelling or atherosclerosis. Moreover, it stimulates the proliferation, differentiation and migration of cells of mesenchymal origin, including fibroblasts, smooth muscle cells and glial cells. It is involved also in the embryonic development of many organs including the brain, lungs, vasculature and kidneys. Finally, PDGFs are involved in wound healing. All PDGFs function as secreted, disulphide-linked homodimers, but only PDGFA and B can form functional heterodimers. Like the other growth factors, also PDGF is synthesized, stored in the platelets alpha granules and released after platelets activation. However, it is also produced by a variety of cells including smooth muscle cells, activated macrophages, and endothelial cells. PDGF family, comprises five different isoforms of PDGF: PDGF-AA, -AB, -BB, -CC and –DD, that activate cellular response through two different receptors PDGFRα and PDGFRβ. These PDGF receptors are classified as a receptor tyrosine kinase (RTK), a type of cell surface receptor. The alpha type binds to PDGF-AA, PDGF-BB and PDGF-AB, whereas the beta type binds with high affinity to PDGF-BB and PDGF-AB. The binding of PDGFs to the PDGFRs induces downstream signalling involving several well-known pathways, e.g. Ras-MAPK, PI3K, PLC-γ pathways and others. Recombinant PDGF is used in medicine to help heal chronic ulcers and in orthopedic surgery and periodontistry to treat bone loss.

1.4 Biomaterials for tissue regeneration

A major goal of tissue engineering is to synthesize or regenerate tissues and organs. Today, this is done by using specific “engineered materials” or biomaterials, which exploit specific and complex physical and biological functions. Combined with growth factors and autologous cells, these biomaterials provide a temporary supporting structure (scaffold), allowing not only three dimensional support of tissue growth and formation but also providing the biological environment needed for cellular growth, proliferation and differentiation. These scaffolds mimic the structure and biological functions of native extra-cellular matrix (ECM), both in terms of chemical composition and physical properties, recapitulating the *in vivo* environment and allowing cells to influence their own microenvironments (*Asghari et al., 2016; Guo et al., 2015*). The ideal scaffold should present the following characteristics: biocompatibility (meaning that it should not provoke any rejection, inflammation, immune responses or foreign body reactions), biodegradability (meaning that it should preferably be absorbed by the surrounding tissues without the necessity of a surgical removal), mechanically resistance and sterilizability, to avoid toxic contaminations without compromising any structural and mechanical properties. Moreover, it should have a porous architecture with an adequate pore size, to allow cell attachment, cell surface interactions, tissue ingrowth and transportation of nutrients and oxygen. Two traditional groups of biomaterials are available (*Stoppel et al., 2015*): natural byopolimers and synthetic polymers. Naturally derived materials include Collagen (*Oliveira et al., 2010*), Alginate, Chitosan (*Chicatun et al., 2013; Hilmi et al., 2013)* Fibrin, Elastin, Fibronectin, Keratin (*Rouse et al., 2010*), Silk (*McNamara et al., 2014; Bellas et al., 2013). Synthetic* materials include Poly(l-lactic acid) (PLA or PLLA) (*Nishio et al., 2012*), Poly(glycolic acid) (PGA), (*Knight et al., 2013; Cao et al., 2010),* Poly(ethylene

glycol) (PEG), Poly(lactic-co-glycolic acid) (PLGA), (*Rahaman et al., 2013*), Polyurethane, Polytetrafluoroethylene (PTFE), Polycaprolactone (PCL) and inorganic materials such as Calcium Phosphates and Hydroxyapatite. Recently the use of platelet preparations such as Platelet Rich Plasma (PRP), Platelet Rich Fibrin (PRF), Platelet Rich in Growth Factors (PRGF) and Concentrated Growth Factors (CGF), has been shown to be effective in promoting the natural processes of wound healing and tissue regeneration.

1.5 Platelet concentrates

Platelet concentrates are blood derivatives (*Prakash et al., 2011; Anitua et al., 2004*), prepared from patient's own blood and containing autologous platelets, growth factors and cytokines involved in the key processes of tissue regeneration, including cell proliferation and differentiation, extracellular matrix synthesis, chemotaxis and angiogenesis. As mentioned before, platelets contain and release a variety of growth factors (VEGF, EGF, FGF, PDGF), coagulation factors, adhesion molecules, cytokines, chemokines and integrins that act on cell growth, proliferation and differentiation. Being autologous, platelet preparations are biocompatible, easy to obtain, safe and without any risk of transmitting infectious disease. According to the classification proposed by Dohan Ehrenfest (*Dohan Ehrenfest et al., 2009*), four main families of platelet preparations can be defined, depending on their cell content and fibrin architecture:

1) Pure Platelet-Rich Plasma (P-PRP) or leukocyte-poor PRP products such as cell separator PRP, Vivostat PRF (*Agren et al., 2014*) or Anitua's PRGF (*Anitua et al., 2007; 2001*): these preparations are free of leukocytes and with a low-density fibrin network after activation;

2) Leukocyte and PRP (L-PRP) products: these preparations contain leukocytes and have a low-density fibrin network after activation;

3) Pure platelet-rich fibrin (P-PRF) or leukocyte-poor platelet-rich fibrin products, such as Fibrinet: these preparations are free of leukocytes and with a high-density fibrin network. They only exist in a strongly activated gel form and cannot be injected or used like traditional fibrin glues;

4) Leukocyte- and platelet-rich fibrin (L-PRF) or second-generation PRP products, such as Choukroun's PRF: these preparations contains leukocytes and have a high-density fibrin network;

Another platelet concentrate, defined as Concentrated Growth Factors (CGF) must be added to this classification.

1.5.1 Platelet Rich Plasma (PRP)

PRP represents the first generation of platelets concentrates, whose pioneer was Marx in 1998. It is an autologous blood product, enriched with platelets, that concentrates a large number of platelets in a small volume of plasma (*Everts et al., 2006*). In fact, according to Marx (*Marx, 2001*), PRP has a platelets concentration of 1000 *109/L in 5ml of plasma, which is 5 times higher than normal baseline whole blood platelet count (200*109/L). In order to obtain PRP, blood must first be drawn from the patient, mixed with an anticoagulant to prevent clotting and then subjected to a two-step gradient centrifugation method. The first centrifugation (called hard spin) allows to separate blood into three distinct layer: acellular plasma or Platelet Poor Plasma (PPP) at the top; a middle layer containing the maximum platelet concentration and the red blood cells (RBCs) at the bottom. The second

centrifugation (called the soft spin) delicately and finely separates the platelets and leukocytes, from the plasma, so obtaining PRP.

PRP has been shown to be effective in promoting the natural processes of wound healing, tissue reconstruction and regeneration (*Gentile et al., 2016; Sengul et al., 2016*). It accelerates endothelial, epithelial and epidermal regeneration, stimulates angiogenesis and enhances collagen synthesis. The potentiality of PRP lies in its ability to incorporate high concentrations of platelet-derived growth factors, as well as fibrin, into the graft mixture. Recently published studies (*Mlynarek et al., 2016; Ahmed et al., 2016; Serraino et al., 2015; Kakudo et al., 2014*) have demonstrated beneficial results with PRP used in a broad range of clinical healing application such as head and neck surgery, otolaryngology, cardiovascular surgery, burns and wound healing, oral and maxillofacial surgery, cosmetic surgery and periodontics. Clinically, PRP is routinely combined with bone substitutes, such as BioOss (an inorganic bovine bone substitute) or demineralized freeze-dried human bone allograft, in oral and maxillofacial surgery. In particular, PRP has been shown to increase the rate of bone maturation and to improve bone density when added to small bony defects or to larger defects in combination with grafting material (*Chen et al., 2014; Faratzis et al., 2012*).

1.5.2 Platelet Rich Fibrin (PRF)

Platelet Rich Fibrin (PRF) consists of an intimate assembly of cytokines, glycanic chains, structural glycoproteins enmeshed within a fibrin scaffold and represents the second generation of platelet concentrates (*Choukroun et al., 2006, Part IV,V*; *Dohan Ehrenfest et al., Part I, II, III, 2006*). The cytokines, glycanic chains, structural glycoproteins can have synergetic effects on tissue healing processes. The PRF

pioneers were Choukroun and colleagues (*Choukroun et al., 2006*), who used it to promote the osseointegration of dental implants. This platelet preparation plays an important role in wound healing and tissue regeneration (*Naik et al., 2013; Kang et al., 2011*). PRF can be used in the form of a platelet gel in conjunction with bone grafts and also as a membrane (*Kobayashi et al., 2012; Jankovic et al., 2012*). PRF preparation is similar to PRP, except that no anti-coagulant is used during blood harvesting. After blood collection, PRF is immediately centrifuged for 10 min to activate the platelets, leading to the initiation of a coagulation cascade. At the end of centrifugation, three different layers are obtained: acellular PPP on top, a PRF clot in the middle and RBCs at the bottom of the test tube. The PRF clot obtained after centrifugation is collected 2 mm below the lower dividing line and the other layers are discarded. The clinical success of the PRF protocol is dependent on a quick collection of blood and its transfer to the centrifuge. Because no anticoagulant is used, the blood sample begins to coagulate almost immediately and a failure to accomplish the quick preparation of PRF could cause a diffuse polymerization of fibrin, which is not ideal for tissue healing. PRF can be considered as a natural fibrin based biomaterial, favourable to the development of a micro vascularisation and is also able to guide epithelial cell migration to its surface. It is used in oral and maxillofacial surgery to improve bone healing in implant dentistry (*Saluja et al., 2011*). Several studies show a PRF efficiency in the treatment of periodontal and periimplant defects (*Pradeep et al., 2016*; *Gupta et al., 2014; Panda et al., 2014; Ranganathan et al., 2014; Desarda et al., 2013; Lee et al., 2012*), cyst cavities (*Pradeep et al., 2016*), gingival recession (*Eren et al., 2014; Aleksić et al., 2010*), sinus floor augmentation procedures (*Ali et al., 2014*; *Xuan et al., 2014*), mandible or maxilla fractures (*Dincă et al., 2014; Kim et al., 2014),* chronic wounds *(Martinez-*

Zapata et al., 2016), articular cartilage defects *(Wu et al., 2016)* and others bone defects.

1.5.3 Platelet Rich in Growth Factors (PRGF)

PRGF is an autologous platelet concentrate prepared using a modified PRP protocol developed by Anitua (*Anitua et al., 2015; 2011*). Unlike PRP, PRGF is optimized to deliver a more sustained release of growth factors. PRGF can create a three-dimensional fibrin scaffold which can be injected into a tissue defect, to maintain the regenerative space and can be used as a scaffold for cells to accomplish tissue regeneration. The leukocyte content of PRGF is eliminated to prevent the pro-inflammatory effects of the proteases and acid hydrolases contained within these cells. PRGF is prepared from a small volume of patient's peripheral venous blood and is collected by a one-step centrifugation with sodium citrate as the anti-coagulant (Endoret System). After activation, PRGF progressively releases a pool of proteins and growth factors, which accelerate soft tissue healing as well as osseous regeneration. It has been used in oral surgery and dentistry to regenerate tissues following cyst enucleations and periapical surgeries (*Singh et al., 2013; Del Fabbro et al., 2009*), to heal tissues following sinus floor elevation treatment (*Anitua et al., 2012; 2009*) and gingival recession treatment (*Lafzi et al., 2011*), in periodontal and periimplant defects (*Birang et al., 2012*). Some studies investigated the clinical potential of PRGF to preserve tissue in tooth extraction sockets prior to dental implant placement. PRGF effect has been showed also *in vitro,* promoting the migration and proliferation of human dental stem cells and gingival fibroblasts (*Anitua et al., 2013; 2011*).

1.5.4 Concentrated Growth Factors (CGF)

CGF, first developed by Sacco, in 2006, is an autologous fibrin network, rich in leukocytes and platelets (*Chen et al., 2016; Honda et al., 2013; Rodella et al., 2011*). CGF also contains autologous osteo-inductive growth factors derived from platelets and an osteo-inductive fibrin matrix. In particular it contains and release some of the principal growth factors involved in tissue regeneration, such as TGF-β1, PDGF-AB, IGF-1, VEGF, TNF-α, BMPs (especially BMP-2 and BMP-7) and BDNF. The kinetic release of these growth factors shows an individual variability (*Borsani, Bonazza et al., 2015*). In fact some growth factors (VEGF and BMP-2) present a slow kinetic release, after six/eight days, others (TNF-α and BDNF) have a fast kinetic release, after one day and others (PDGF-AB, TGF-β1 and IGF-1) have a constant release. Similar to PRF, CGF is created using a one-step centrifugation method, but it requires a special programmed centrifuge (*Medifuge MF200, Silfradent srl, Forli, Italy*), which uses plastic tubes, coated with silica particles and without the addition of exogenous substances. The final blood product is separated into three layers: (1) the upper layer, representing the liquid phase of plasma named platelet poor plasma (PPP); (2) the lower layer, representing red blood cells (RBC) because of mainly contains erythrocytes; (3) the middle layer, representing the solid CGF consisting in three parts: the upper white part (WP), the downer red part (about 0,5 cm from RBC) and the middle “buffy coat” part (interface between white and red part), (*Bonazza et al., 2016; Borsani,Bonazza et al., 2015*). Morphologically, as regards blood cells localization in CGF, it was shown that leukocytes were localized principally in the buffy coat (BC) but also scattered around it, especially in the red part (RP) of CGF; whereas the erythrocytes were present only in the RP (*Borsani, Bonazza et al., 2015*). Immunohistochemistry with the platelet marker CD61, showed that platelets

were localized in the BC, forming aggregates. Moreover, CGF presents a different fibrin architecture moving from the BC to the WP of the CGF. In particular, near the BC the fibrin network of the WP, appeared strictly compact, with thight meshes, while far from the BC appeared less compact, with larger meshes (*Borsani, Bonazza et al., 2015*).

1.5.4.1 CGF in tissue regeneration

Being the most recent platelet concentrate developed, there are no many studies in literature regarding the use of CGF in tissue regeneration. Till now, the use of CGF has been reported in the field of dental implantology, oral and maxillofacial surgery, in head and neck surgery, otolaryngology, cardiovascular surgery, burns and wound healing, cosmetic surgery and periodontics ligament diseases. In particular CGF has been used successfully in maxillary sinus augmentation, in order to facilitate new bone formation and reduce healing time (*Chen et al., 2016; Kim et al., 2014; Sohn et al., 2009*) and in the treatment of multiple gingival recessions (*Doğan et al., 2015*). Its beneficial effect has been reported also *in vitro* showing that CGF enhances the migration and proliferation of human dental stem cells and gingival fibroblasts (*Yu et al., 2014*), Schwann cells (*Qin et al., 2016*) and Rat Bone Marrow cells (*Durmuşlar et al., 2016;Takeda et al., 2015*). Moreover this autologous platelet concentrate promotes bone regeneration and repair in rat calvarial bone defects *in vivo* (*Takeda et al., 2015)*, especially when used togheter with bone marrow derived stromal cells (BMSCs), (*Honda et al., 2013).*

1.6 Trace elements in tissue regeneration

Trace Elements are essential for normal growth and development of skeleton in humans and animals. Although they are minor building components in teeth and bone, they play important functional roles in bone metabolism and bone turnover. These elements include: Magnesium, Selenium, Zinc, Copper, Silicon and many others. Magnesium enhances bone turnover through the stimulation of osteoclastic function (*Costa et al., 2016; Hussain et al., 2014)*. Zinc regulates secretion of calcitonin from thyroid gland and influences bone turnover. It is a cofactor in a number of intracellular enzymatic reactions pertaining to wound healing. It is also an antioxidant, confers resistance against epithelial apoptosis and has significant antibacterial properties. Zinc doped Calcium Phosphate scaffolds or biopolymer scaffolds showed improved osteogenic differentiation. When incorporated into phosphate-based glasses (*Chou et al., 2013; 2015*), bone cell adhesion and proliferation were also enhanced. Moreover it has been showed that Zinc promotes the growth and osteogenic differentiation of mesenchymal stem cells (*Chou et al., 2015*). Selenium has strong antioxidant properties (*Bajpai et al., 2011*) and has a beneficial role also in tissue repair and regeneration (*Wang et al., 2013*). Copper is an essential trace element abundantly presents in liver tissue and it is known for its stimulatory effect on angiogenesis, in endothelial cells. It functions as a cofactor and is an important component in the structural and catalytic properties of many enzymes, such as superoxide dismutase, which plays an antioxidant action, protecting the body against the harmful effect of superoxide. Its use together with biosynthetic materials improves angiogenic and osteogenic capacity for repairing osseous defects (*Zhao et al., 2014*). Silicon is another essential trace element that

plays an important role in tissue regeneration as described in detail in the following paraghraphs.

1.6.1 Silicon: chemistry and structure

Silicon (Si) is a chemical element, in the carbon family (Group 14 of the periodic table), with an atomic weight of 28. It is classified as a semiconductor with electrical properties that are intermediate between metal and non-metal elements. The name Silicon derives from the Latin silex or silicis, meaning “flint” or “hard stone.” Amorphous elemental Silicon was first isolated and described as an element in 1824 by Jöns Jacob Berzelius, a Swedish chemist. This trace element, represents the second most abundant element in the Earth's crust (28 %), after Oxygen (47%), (Figure 4) but it is rarely found in its elemental form due to its great affinity for Oxygen, forming silica (SiO_2) and silicates, which at 92%, are the most common minerals.

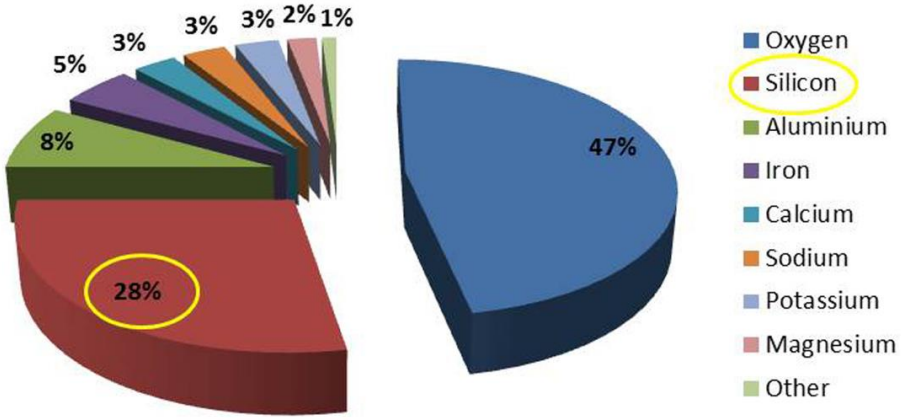

Figure 4: most abundant element in the Earth's crust

In particular, SiO_2 is the most studied chemical compound following water and the most important Si-containing inorganic substance (Figure 5). The Silicon dioxide form is the major component of sand, granite, quartz and other types of rocks, clays and gems in the Earth's crust. Quartz (12%) and the alumino-silicates are the most prevalent silicates. These are present in igneous and sedimentary rocks and soil minerals and are highly stable structures that are not readily broken down except with extensive weathering. Thus natural levels of soluble (available) silica are low.

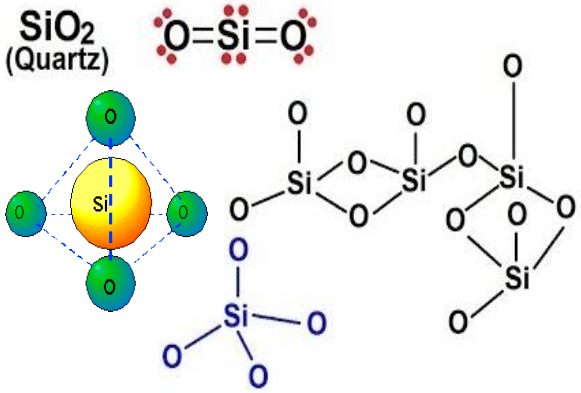

Figure 5: SiO_2 structure

1.6.2 Silicon sources

Human are exposed to numerous sources of Silicon including dust, pharmaceuticals, cosmetics and medical implants and devices, but the major and most important Si source for the majority of the population is the diet (*Jugdaohsingh et al., 2002*). Si average daily intake ranges from about 20 mg/day to 50 mg/day in Western countries. Higher intakes (104 mg/day – 204 mg/day), have been reported in China and India, where plant-based foods may form a more predominant part of the diet. Mean Si intakes in men (33 mg/day) are significantly higher than in women (25

mg/day). In children the major Si source is from cereals (68% of total dietary intake), whereas the major source in adult males is from beer ingestion (44%). Silicon intake decreases with age.

A) Food sources

Foods derived from plants rather than animals provide the highest sources of dietary Si (*Pennington, 1991*), because certain plants, especially cereals, are Si accumulators (*Epstein, 1999*). Plants take up and accumulate Si from soil and soil solutions that becomes incorporated as a structural component conferring strength and rigidity to stalks, for example, in grasses and cereals and also in some plants such as horsetail (Equisetum arvense) which is known to be one of the strongest accumulators of Si among higher terrestrial plants (*Law et al., 2011; Sapei et al., 2007*). Plants produce biogenic (phytolithic) silica which is often associated with the polysaccharide/carbohydrate components of the cell wall. Foods rich in Silicon include: cereals, especially oats, barley, white wheat flour and some fractions of rice (*Robberecht et al., 2009*). Up to 50%, of the Si is present in the hulls and husks. Rice hulls, for example, contain 110 mg Si/100g, and during industrial treatment these are removed which reduces Si in the refined foods. However, grain products such as breakfast cereals, flour and bread, biscuit, rice, pasta, etc., are still high dietary sources of Si (*McNaughton et al., 2005; Powell et al., 2005*). Vegetables like beans (2.5 mg Si/100 g), spinach, carrots, radish and beetroot, are reasonably high in Si (*Jugdaohsingh et al., 2002*). Fruits generally contain low levels of Si, except for bananas and from which Si absorption appears to be unusually low (*Jugdaohsingh et al., 2002*), suggesting that the form of Si is poorly digestible in this food source. Dried fruits (e.g. raisins) and nuts also have a relatively high level of Si but their intakes are

generally low. Sugar cane also actively takes up Si and refined and unrefined sugars are also high in Si.

B) Non-food sources

Silicon may also be ingested as a supplement in tablet, capsule, gel and solution forms and as pharmaceuticals, since silicates are widely used as active components of anti acids and anti diarrhoeals (e.g. aluminium or magnesium trisilicates, etc.), or as an inert component (excipient) in many medications (*Lomer et al., 2004*). The absorption of Si from these preparations has not been well characterized, although there have been a few studies (*Van Dyck et al., 1999; Cefali et al., 1995*). Soil adherent to vegetables is a further source of ingested Si, but absorption is likely to be low as mineral silicates are expected to be stable and not broken down in the gastrointestinal tract. Humans may also come into contact with Si through exposure to siliceous dust, talc, etc., but this usually constitutes a minor exposure and dissolution to form available orthosilicic acid is probably minimal. Apart from these sources, medical and dental implants used in the replacement and repair of tissue, bone or dentine, or silicone tubing used in haemodialysis, for example, can also be sources of Si although these are also minor for the majority of the population. Silicon is also present in cosmetics, creams and toothpastes but gastrointestinal or dermal absorption from these is minimal because the silicates are particulate and poorly broken down to release the active (orthosilicic acid) moiety. Phytolithic silica may be present, as a contaminant, in facial scrub and shampoos as often these are plant based, while silicones may be present in some hand and nail creams and in nail varnish.

C) Beverages

Silicon is highly available from drinking water (*Jugdaohsingh et al., 2002*) and its concentration is initially dependent upon the geology of the source of water (*Perry & Keeling-Tucker, 1998; Birchall & Chappell, 1980*). Water permeating through 'old rock' contains less Si compared with that from 'young rock' areas. In the UK for example, Si concentrations are low (0.2-2.5 mg/L) in the north and west of Britain ('highland' Britain), where the rocks are 'old' and well-weathered and the water is naturally soft. In contrast, Si levels are much higher (2.8-14 mg/L) in the south and east of Britain ('lowland' Britain) from the weathering of 'young rocks'; the water is naturally hard as it is high in dissolved solids and is also alkaline. The Si concentration of European mineral waters is within a similar range (4-16 mg/L) to lowland drinking waters and their pH is typically around neutral, or slightly above. Recently, however, higher levels (30-40 mg/L) have been reported in Spritzer and Fiji mineral waters, from natural sources in Malaysia and Fiji respectively. Drinking water and other fluids provides the most readily bioavailable source of Si in the diet, since Si is principally present as Orthosilicic Acid (OSA, H_4SiO_4) and fluid ingestion can account for ≥ 20% of the total dietary intake of Si. High Si levels are found also in beer (*Sripanyakorn et al., 2004*), which is made from macerated whole-grain barley from which H_4SiO_4 is released into the beer.

1.6.3 Silicon absorption and excretion

The Si bioavailability depends on the solubility of the compound or speciation concerned. There is a lack of reliable data about the metabolism of Si in humans. This is partly due to the fact that there are many different forms of dietary Silicon, all with different absorptions. Silicic acid is the bioavailable form, especially as mono

(=ortho) and di silicic acid. It is easily absorbed from the gastro-intestinal tract (50–80%). In the gastrointestinal tract Si, as OSA (H_4SiO_4), is available from fluids (20-30%) and from silica in solid foods (70-80%), which is hydrolyzed to H_4SiO_4. The average absorption of daily Si intake is less than 50%. The absorption is facilitated by aquaporins (a family of small channel proteins present in the intracellular membranes, where they facilitate the transport of water and/or small neutral solutes like urea, boric acid, silicic acid). In humans several aquaporins for OSA are identified (AQP3, AQP7, AQP9 and AQP10), (*Garneau et al., 2015*). Another possibility for silicon uptake by the body is transdermal absorption. Lassus (*Lassus, 1997*) showed the effects of oral and topical treatment of aged skin by a silicic gel. Because of the combined method of administration the effects of dermal absorption alone remain unclear. After absorption, OSA is readily filtered by the renal glomerulus, because it does not associate with plasma proteins and hence much absorbed Si is rapidly excreted into urine *(Reffitt et al., 1999; Jugdaohsingh et al., 2002*). Thus, urinary excretion of Si is a good surrogate marker of Si absorption (*Calomme & Berghe, 1997; Reffitt et al., 1999; Van Dyck et al., 1999*). It is not clear how much of the absorbed Si is retained in tissues, but it is likely to be small (≤ 10%), (*Popplewell et al., 1998*). Although it has been suggested that Si is poorly absorbed from food (*Van Dyck et al., 1999*), Jugdaohsingh and colleaugues (*Jugdaohsingh et al., 2002*) have shown that much of the Si in food is broken down into the monomeric form in the gastrointestinal tract and then absorbed. Thus serum Si levels significantly increase after ingestion of foods rich in Si, peaking 100–120 min after ingestion (*Jugdaohsingh et al., 2002*). A mean of 41 ± 36% of ingested Si was excreted in urine (i.e. at least that amount was absorbed), following ingestion of Si-rich foods. Indeed, the Si content of food correlates significantly with urinary Si excretion following ingestion of the food, suggesting that for most foods their Si

content can be used as an approximate indicator of their absorption (*Jugdaohsingh et al., 2002*). However, from some foods, such as root vegetables and bananas, little Si appears to be absorbed (*Jugdaohsingh et al., 2002*), although further work is required to confirm and explain this.

1.6.4 Silicon tissue distribution

The biological importance of Silicon, is closely related to its bio distribution in the human organism. Silicon, after Iron and Zinc, is the third most abundant trace element in the human body (at ~140–700 mg). The highest Si concentration is in connective tissues, especially aorta, trachea, tendon and in fast growing cells such as hair, nails, bone and skin cells. The reason for their high Si content is proposed to be the binding of Si to glycosaminoglycans and their protein complexes in connective tissues. Silicon is also found at lower levels in parenchymal tissues, such as liver, heart, muscle and lung (*Carlisle, 1982*) and it exists in the OSA form, not associated with proteins, in blood (*Adler & Berlyne, 1986*).

1.6.5 Biological role of Silicon: the Prolylhydroxylase

Silicon would seem to act on the enzyme Prolyl hydroxylase, whose catalytic action is important in the synthesis of collagen and glycosaminoglycans, allowing the conversion (internal to the structure of collagen) of L-Proline amino acid into Hydroxyproline (Figure 6). This biological catalyst plays a key role in the development of joints, bones, skin and skin appendages and therefore Silicon intake stimulates the synthesis of Collagen, leading to an harmonious development of connective and other tissues.

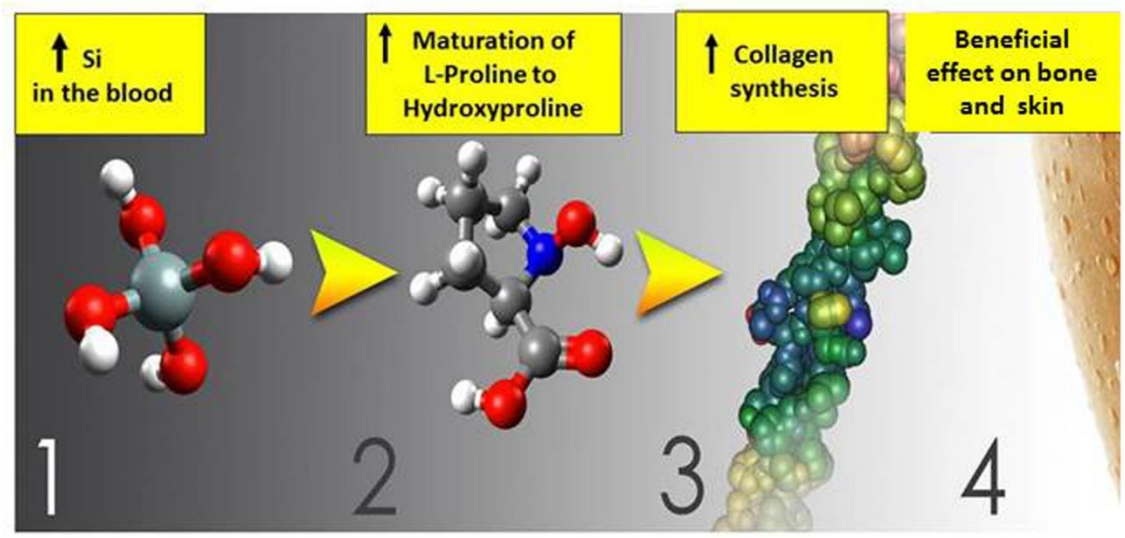

Figure 6: Silicon biological effect on the enzyme Prolylhydroxylase.

In the figure above the possible Silicon mechanism of action is showed: an increase of Silicon concentration in the blood (1) is progressively followed by an increase in the maturation of L-proline to Hydroxyproline (2); this leads to a more efficient synthesis of Collagen (3) that, in the space of a few months, leads to an improvement in mechanical properties of bones, skin and skin appendages (4).

All this suggests that, acting on the synthesis of Collagen and glycosaminoglycans, we can improve the mechanical properties and appearance of various tissues and organs, including the skin.

1.6.6 Silicon in tissue regeneration

Several studies, performed both *in vivo* and *in vitro* (*Kim et al., 2009; Bae et al., 2008; Calomme et al., 2006; Jugdaohsingh et al 2004; Refitt et al., 2003*), suggest that dietary Si consumption is beneficial for different organs and tissues, such as: bone and cartilage, brain, skin, nails and hair, cardiovascular system and immune system.

1.6.6.1 Bone and other connective tissues

Higher Si levels are reported to be associated with healthy connective tissues (e.g. aorta, bone, trachea, tendon) and especially their connective tissue components (i.e. collagen and elastin), compared to non-connective tissues (e.g. liver, kidney, spleen). The Si concentration in connective tissues is suggested to decrease with age and with disease progression, based upon data for the human aorta (*Charnot et al., 1971*). As concern bone tissue, several studies conducted in ovariectomized rats (*Kim et al., 2009; Calomme et al., 2006; Seaborn et al., 2002; Hott et al., 1993*), showed a Si beneficial effect on bone metabolism, suggesting its preventive or therapeutic role in different bone diseases like osteoporosis. The ovariectomized rat is the standard model for post menopausal bone loss and the studies conducted using this model, showed that consumption of a diet rich in Si, stimulated bone growth and mineralization, especially of long bones such as femur and tibia. On the contrary, Si deficiency, resulted in an increased degradation of bone and cartilage, impaired bone mineral composition and decreased activity of bone enzymes, such as alkaline phosphatase (ALP), with a consequent higher risk of fractures or bone diseases (*Rodella, Bonazza et al., 2014*). Other studies (*Kayongo et al., 2008; Sahin et al., 2006; Carlisle 1980,1976,1972),* on growing animals showed that Si dietary supplementation improved bone quality, increasing bone mineral density and reducing bone fragility. Studies on humans, especially on pre and post-menopausal women (*Macdonald et al., 2012; Li et al., 2010; Spector et al., 2008; McNaughton et al., 2005; Jugdaohsingh et al., 2004*), showed that 40 mg of Si daily intake is associated with greater bone mineral density, reducing the risk of bone fractures. In *vitro* studies have also been conducted to determine the mechanism of Si effect on bone. The *in vitro* effects of OSA (0-50 μM) was investigated (*Refitt et al., 2003*)

using the human MG-63 osteosarcoma cell line and an immortalized human early osteoblastic cell line (HCC1), as osteoblast model, showing that physiological concentrations of OSA (10-20 μM), stimulated Collagen type I synthesis and enhanced osteoblastic differentiation in both the cell lines used. In an another work, (*Kim et al., 2013*), the Si effect, in the form of Sodium Metasilicate, on the MC3T3 murine cell line, was investigated, showing an increase in bone formation and mineralization. So all these results suggest that Si acts as a biocatalyst which can be used in the preventive and healing therapies of disorders related to the joints and the skeleton, such as osteoarthritis and post-menopausal osteoporosis. The loss of bone mass is closely related also to an increase of vascular calcification, with a higher risk of developing vascular diseases, such as atherosclerosis. In fact, some epidemiological studies have demonstrated the existence of a positive correlation between the increase in arterial calcification and the reduction of bone mineral density (*Celik et al., 2010; Persy et al., 2009; Park et al., 2008*). Moreover, further evidence of Silicon role in bone is provided by *in vivo* and *in vitro* studies with Silicon containing implants and ceramics such as Si-substituted hydroxyapatite and silica-based bioactive glass (*De Godoy et al., 2015; Shadjou et al., 2015; Kim et al., 2015*). Silica on these materials undergoes partial dissolution, forming an amorphous Si layer and the dissolved Silicon is involved in gene upregulation, osteoblast proliferation and differentiation, type I collagen synthesis and apatite formation. Based on the evidences of Si beneficial properties on bone health, artificial Si scaffolds have been generated, especially during the last years.

1.6.6.2 Brain

Apart from its physiological role in bone and cartilage formation, Silicon seems to be an essential element also for the brain, in which dietary Si provides protection against aluminum (Al) accumulation and consecutive oxidative damage (*Domingo et al., 2011; Belles et al., 1998*). This effect has been associated with protection against Alzheimer's disease (*Davenward et al., 2013*) because Al interferes with glutamatergic neurotransmission causing neurotoxicity and death of nitrergic neurons, by inducing glutamatergic cytotoxicity and consequently an overproduction of Nitric Oxide (NO) (*Llansola et al., 1999*). Several studies (*Foglio et al., 2012; Exley, 2006*) suggest that dietary Si supplementation might have a protective role, reducing the Aluminium gastrointestinal adsorption and increasing its excretion via the urine, probably by interacting with filterable Al in renal tubules, forming hydroxylaluminosilicates and impeding the metal re-absorption. In particular Si mantains the number of nitrergic neurons and their expression of nitrergic enzymes at physiological levels. Silicic acid has also been found to induce down-regulation of endogenous antioxidant enzymes associated with aluminum administration.

1.6.6.3 Cardiovascular system

Some evidences suggest a positive correlation between Silicon consumption and vascular homeostasis (*Schwarz et al., 1977*). In particular, it seems that dietary Si intake improves the cardiovascular system, being essential to the structural integrity, elasticity and permeability of the arteries and exerting a protective and preventive anti-atherosclerotic effect. The exact mechanism of action is not fully known and its understanding requires further in-depth investigation. However, some recent studies (*Buffoli et al., 2013*) have shown that, at the vascular level, Si results in an increase

of some vasoactive molecules involved in the processes of oxidative stress, the endothelial nitric oxide synthase (eNOS) and aquaporin-1 (AQP1) and could therefore act as a protective factor against vascular alterations.

1.6.6.4 Skin, hair and nails

To date, there has been little research concerning the effects of dietary Si intake, on skin and its appendages (hair, nails). As regards skin, it is suggested that Si is important for optimal synthesis of Collagen and for activating the hydroxylation enzymes, improving skin strength and elasticity. It was shown that physiological concentrations of OSA stimulate fibroblasts to secrete Collagen type I *(Refitt et al., 2003).* The first report about Si and skin dated back to 1958 (*Fregert, 1958*). The aim of the study was to quantify Si levels in human tissues (especially skin) and in some human internal organs (aorta, kidneys), derived from cadavers and surgical materials. The highest Si concentration was found in hair and epidermis, particularly in the cornified layer. On the contrary, the amount of Si in patients with skin diseases, like psoriasis and exfoliative dermatitis, was lower than in healthy subjects, probably because these pathologies are characterized by incomplete keratinization process. Subsequently, Barel and colleagues (*Barel et al., 2005*) investigated the influence of Si supplementation, in the form of OSA stabilized with Choline (ch-OSA), on skin and its appendages, in a double-blind placebo controlled study. It was observed that treatment with ch-OSA determined a significant improvement in skin thickness and turgor and in hair and nails brightness. In 2007, Wickett and collegues (*Wickett et al., 2007*), conducted another study, to evaluate the influence of ch-OSA oral intake on hair structure and morphology. In this randomized, double blind placebo-controlled study, 48 women with fine hair were given 10 mg of ch-OSA a day or placebo for

nine months. At the end of the study, the researchers concluded that the women taking bioavailable silica had thicker hair because of increased elasticity and tensile strength. These findings have led the researchers to conclude that Silicon has a positive role also on the mechanical properties of the skin surface as well as on the strength of its appendages like hair and nails.

2. AIM OF THE STUDY

The aim of the present study was to evaluate the *in vitro* effect of three different treatments (Silicon, in the soluble form of Sodium Orthosilicate, CGF and Sodium Orthosilicate together with CGF), on the growth, proliferation and metabolic activity of three different human cell lines: human osteoblasts cells (HOBs), human fibroblasts cells (NHDF - Normal Human Dermal Fibroblasts) and human endothelial cells (HUVEC - Human Umbilical Vein Endothelial Cells).

3. MATERIAL AND METHODS

3.1 Human cell lines

For *in vitro* experiments with Sodium Orthosilicate, CGF and Sodium Orthosilicate togheter with CGF we used three different cryopreserved human cell lines (Figure 7) that are described in detail below:

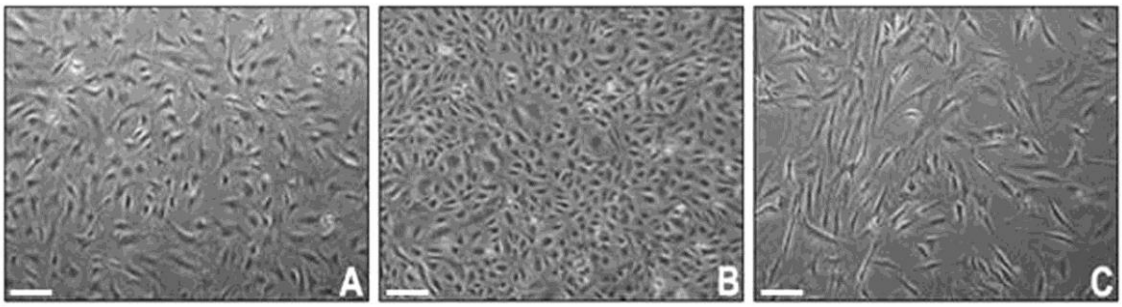

Figure 7: Human cell lines used for *in vitro* experiments: A) Normal Dermal Human Fibroblasts (NHDF); B) Human Umbilical Vein Endothelial Cells (HUVEC); C) Human Osteoblast cells (HOBs). Bar = 80 µm.

A) Normal Dermal Human Fibroblasts – NHDF: fibroblasts are the main cells of connective tissue responsible for the production of collagen and mucopolysaccharides. Furthermore, these cells are specialized in the deposition of extra cellular matrix and in the maintenance, degradation and rearrangement of its structure, through the production of protein molecules including laminin and fibronectin. NHDF grow adherent in a specific medium named Fibroblast Growth medium (FGM) enriched with serum and growth factors. Morphologically NHDF show an elongated shape.

B) Human Umbilical Vein Endothelial Cells – HUVEC: these cells are primary endothelial cells obtained by enzymatic digestion from the vein of the umbilical cord and make up the inner lining of all blood vessels and lymph vessels. Therefore, they are used as a laboratory model system for the study of the biology and pathophysiology of the endothelium and its interactions with other cell types and matrix components. They synthesize and secrete activators as well as inhibitors of both the coagulation system and the fibrinolysis system in addition to mediators that influence the adhesion and aggregation of blood platelets. Endothelial cells also release molecules that control cell proliferation and modulate vessel wall tone. HUVEC grow adherent in a specific endothelial medium named Endothelial Growth Medium (EGM), supplemented with serum and growth factors. Morphologically, these cells show a round shape.

C) Human Osteoblast cells – HOBs: Human osteoblasts are a highly specialized cell type, of mesenchymal origin, involved in bone formation and remodelling. *In vivo* they produce the osteoid, an extracellular matrix rich in Collagen type 1. In the course of their natural maturation process into osteocytes, called osteogenesis, they become embedded in the bone matrix and stop proliferating. They grow adherent in a specific medium named Osteoblast Growth Medium (OGM) supplemented with serum and specific growth factors. These cells show an elongated shape and are morphologically similar to NHDF.

3.2 Cell cultures

The cryopreserved cells were thawed following the specific protocol and cultured until confluence in ther specific complete medium and then subcultured in order to have a sufficient number of cells to perform *in vitro* experiments.

3.3 Cell treatments

The three different human cells lines were subjected to the following treatments:

1) Sodium Orthosilicate

2) CGF

3) Sodium Orthosilicate + CGF

3.3.1 Sodium Orthosilicate preparation

For the *in vitro* experiments we used Silicon in the form of Sodium Orthosilicate (Na_4SiO_4), (*Alfa Aesar,Germany*) because readily soluble in water, producing an alkaline solution. A stock solution of Na_4SiO_4 at a final concentration of 0,1 M, was prepared. The powder was weighted using an analytical balance and then it was dissolved in sterile water using heating plate and a magnet. The pH solution was checked using a calibrated pH meter. Nitric Acid 10M (*VWR International, Milan*) was added to lower the pH solution. Once prepared, the stock solution was filtered using a 0.2 µm syringe filter, under a laminar flow cabinet and two different concentrations of Sodium Orthosilicate (0,5 and 1mM) were prepared in cell culture media by serial dilution of the 0,1 M stock solution.

3.3.2 CGF preparation

For the experiments, the whole blood was collected by piercing a superficial venous blood vessel with a 21-gauge needle from a total of 3 healthy adult volunteers of Caucasian ethnicity consisting of 1 men (V1) and 2 women (V2,V3), aged 28 to 53 years. Exclusion criteria were a systemic disorder, smoking, infection, non-steroidal anti-inflammatory drug use, a hemoglobin level of < 11g/dl or a platelet concentration of < 150000 mm^3. To avoid variances from the different subjects, blood was collected from the three volounteers during all the study. Once collected, the samples were always immediately processed in order to obtain CGF.

The CGF was produced as follow: 9 mL of blood was drawn in each sterile Vacuette tubes (*Greiner Bio-One, GmbH, Kremsmunster, Austria*), coated with silica microparticles as serum clot activator. These tubes were then immediately centrifuged in a special machine (*Medifuge MF200, Silfradent srl, Forlì, Italy*) using a program with the following characteristics: 30'' acceleration, 2' 2,700 rpm, 4' 2,400 rpm, 4' 2,700 rpm, 3' 3,000 rpm, and 33'' deceleration and stop. At the end of the process, three blood fractions were identified (Figure 8A): (1) the upper layer, representing the liquid phase of plasma named platelet poor plasma (PPP); (2) the lower layer, representing red blood cells (RBC) because of mainly contains erythrocytes; (3) the middle layer, representing the solid CGF consisting into three parts: the upper white part (WP), the downer red part (RP), about 0,5 cm from RBC and the middle "buffy coat" part (BC), interface between WP and RP (Figure 8C). At the end of the centrifugation process, CGF was removed from each tube, using sterile tweezers and placed on the surface of sterile petri dish, under a laminar flow cabinet. Whole solid CGF was obtained cutting the lower part of RBC, using sterile scissors (Figure 8B). Subsequently, each whole CGF was processed in relation to the experiment.

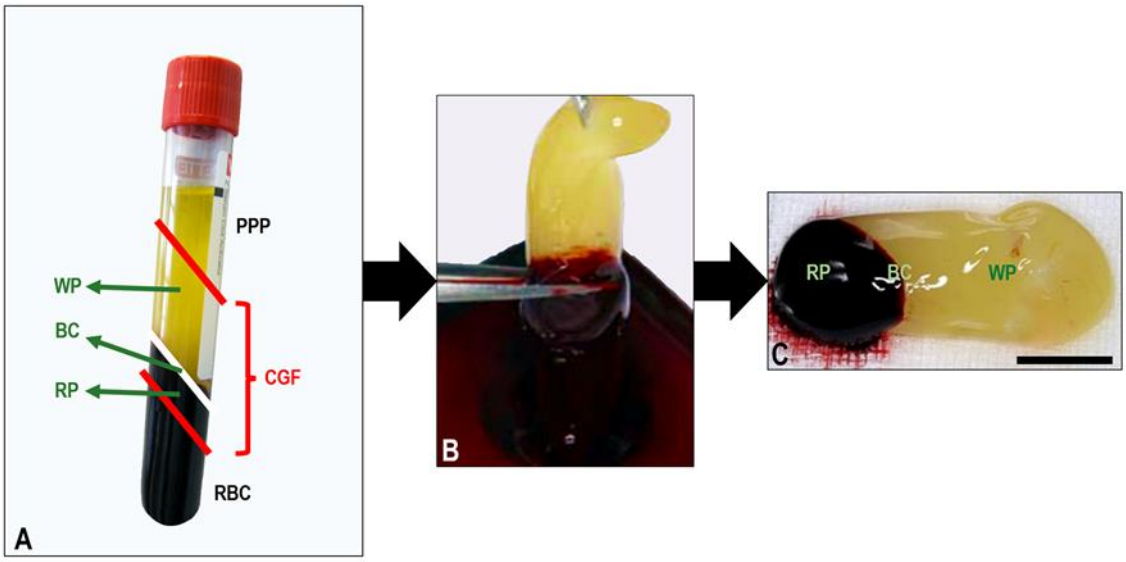

Figure 8: Blood sample after CGF protocol of centrifugation. Three layers are obtained: PPP, upper layer; CGF, middle layer; RBC, lower layer (A). CGF was removed from the tube, using sterile tweezers and RBC cut at 0,5 cm under buffy coat (BC) (B). The whole CGF consists into three parts: the upper white part (WP), the downer red part (RP) and the middle BC part (C). Bar = 1 cm. (modified by *Borsani, Bonazza et al., 2015*).

3.3.3 Cell treatment with Sodium Orthosilicate

1) NHDF

NHDF were cultured in Fibroblast Growth Medium (*FGM; Lonza, Walkersville MD, USA*) constituted by Fibroblast Basal medium (*FBM; Lonza, Walkersville MD,* USA) supplemented with gentamicin/amphotericin B (antibiotic/antifungal) and growth factors (*rhFGF-B, insulin, fetal bovine serum - all from BulletKits®, Lonza, Walkersville MD, USA*), at 37 °C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence, changing the medium every 2 days. NHDF from third and sixth passage were used in the experiments. At confluence, NHDF were

passaged and seeded at a final density of 10000 cell/cm^2, in 6-well culture plates (*Sarstedt, Nuembrecht, Germany*) and starved in FBM for 24 hours, before stimulation. Then the medium was removed and the cells were subjected to the following treatments for 72 hours: 1) **only FGM** (which represents the control); 2) **FGM + Na_4SiO_4 0,5 mM;** 3) **FGM + Na_4SiO_4 1 mM.**

2) HUVEC

HUVEC (*pooled cells; Lonza, USA*) were cultured in Endothelial Growth Medium (*EGM; Lonza, Walkersville MD, USA*) constituted by Endothelial Basal Medium 2 (*EBM2; Lonza, Walkersville MD, USA*) supplemented with gentamicin/amphotericin B (antibiotic/antifungal) and growth factors (hFGF, VEGF, IGF-1, hEGF, fetal bovine serum, all from EGM-2 Single Quot®; Lonza, Walkersville MD, USA) at 37 °C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence, changing the medium every 2 days. Experiments were performed using HUVEC between third and sixth passage. At confluence, HUVEC were passaged and seeded, at a final density of 10000 cell/cm^2, in 6-well culture plates (*Sarstedt, Nuembrecht, Germany*) and starved in EBM2 for 24 hours, before stimulation. Then the medium was removed and four different treatments were tested for 72 hours: 1) **only EGM** (which represents the control); 2) **EGM + Na_4SiO_4 0,5 mM**; 3) **EGM + Na_4SiO_4 1 mM.**

3) HOBs

HOBs (*cryopreserved cells; Promocell, Germany*), were cultured in Osteoblast Growth Medium (*OGM; Promocell, Heidelberg, Germany*) constituted by Osteoblast Basal Medium (*OBM; Promocell, Heidelberg, Germany*) supplemented with gentamicin/amphotericin B (antibiotic/antifungal) and SupplementMix (*OGM Supplement Mix; Promocell, Heidelberg, Germany*) containing growth factors (not

specified by the manufacturer) at 37 °C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence, changing the medium every 2 days. Experiments were performed using cells between third and sixth passage. At confluence, HOBs were passaged and seeded, at a final density of 10000 cell/cm^2, in 6-well culture plates (Sarstedt, Nuembrecht, Germany) and starved in OBM for 24 hours, before stimulation. Then the medium was removed and four different treatments were tested for 72 hours: 1) **only OGM** (which represents the control); 2) **OGM + Na_4SiO_4 0,5 mM**; 3) **OGM + Na_4SiO_4 1 mM.**

3.3.4 Cell treatment with CGF

1) NHDF

NHDF were cultured in FGM, in a humidified atmosphere until they reached about 80% confluence, changing the medium every 2 days. NHDF from third and sixth passage were used in the experiments. At confluence, NHDF were passaged and seeded, at a final density of 10000 cell/cm^2, in 6-well culture plates and starved in FBM for 24 hours, before stimulation. Then the medium was removed and four different treatments were tested for 72 hours: 1) **only FBM**; 2) **only FGM**; 3) **FBM + CGF**; 4) **FGM + CGF.**

2) HUVEC

HUVEC were cultured in EGM, at 37 °C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence, changing the medium every 2 days.

Experiments were performed using HUVEC between third and sixth passage. At confluence, HUVEC were passaged and seeded, at a final density of 10000 cell/cm^2,

in 6-well culture plates and starved in EBM2 for 24 hours, before stimulation. Then the medium was removed and four different treatments were tested for 72 hours: 1) **only EBM**; 2) **only EGM**; 3) **EBM + CGF**; 4) **EGM + CGF.**

3) HOBs

HOBs were cultured in OGM, at 37 °C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence, changing the medium every 2 days. Experiments were performed using cells between third and sixth passage. At confluence, HOBs were passaged and seeded, at a final density of 10000 cell/cm^2, in 6-well culture plates and starved in OBM for 24 hours, before stimulation. Then the medium was removed and four different treatments were tested for 72 hours: 1) **only OBM**; 2) **only OGM**; 3) **OBM + CGF**; 4) **OGM + CGF.**

3.3.5 Cell treatment with Sodium Orthosilicate and CGF

1) NHDF

NHDF were cultured in FGM, at 37 °C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence, changing the medium every 2 days. NHDF from third and sixth passage were used in the experiments. At confluence, NHDF were passaged and seeded, at a final density of 10000 cell/cm^2, in 6-well culture plates and starved in FBM for 24 hours, before stimulation. Then the basal medium was removed and three different treatments were tested for 72 hours: 1) **FGM + CGF**; 2) **FGM + CGF + Na_4SiO_4 0,5 mM**; 3) **FGM + CGF + Na_4SiO_4 1 mM.**

2) HUVEC

HUVEC were cultured in EGM, at 37 °C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence, changing the medium every 2 days.

Experiments were performed using HUVEC between third and sixth passage. At confluence, HUVEC were passaged and seeded, at a final density of 10000 cell/cm^2, in 6-well culture plates and starved in EBM2 for 24 hours, before stimulation. Then the basal medium was removed and three different treatments were tested for 72 hours: 1) **EGM + CGF**; 2) **EGM + CGF + Na_4SiO_4 0,5 mM**; 3) **EGM + CGF + Na_4SiO_4 1 mM.**

3) HOBs

HOBs were cultured in OGM, at 37 °C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence, changing the medium every 2 days. Experiments were performed using cells between third and sixth passage. At confluence, HOBs were passaged and seeded, at a final density of 10000 cell/cm^2, in 6-well culture plates and starved in OBM for 24 hours, before stimulation. Then the basal medium was removed and three different treatments were tested for 72 hours: 1) **OGM + CGF**; 2) **OGM + CGF + Na_4SiO_4 0,5 mM**; 3) **OGM + CGF + Na_4SiO_4 1 mM.**

In all the three cell lines used, whole CGFs were not put into direct contact with cells but each whole CGF was placed into a sterile transwell insert (*ThinCertTM cell culture inserts, Greiner Bio-One, Austria*) with a semi-permeable membrane at the bottom (pores of 0,4 µm) and inserted into the 6-well culture plates (an insert in each well) for 72 hours.

The final *in vitro* effect of the different treatments, on cell growth and proliferation was evaluated both by performing a simple cell count, using an automated cell counter (*Scepter™ 2.0 Cell Counter, Millipore*) and by evaluating the expression of the intracellular proliferation marker Ki-67, using FACS. Moreover, we evaluated also the expression of other cell markers such as Collagen type I (Col I), Osteopontin (OPN), Vascular Endothelial Growth Factor (VEGF), endothelial Nitric Oxide oxide synthase (eNOS), performing immunohistochemical analyses on fixed cells.

3.4 FACS analysis

Cells were detached with the Trypsin (0,025%)/EDTA (0,01%) solution (*Promocell, Heidelberg, Germany*) and centrifuged at 1000 rpm for 5 minutes. After removing the supernatant, pellet was re-suspended in the appropriate culture medium. Cell suspension (100-200 µl), was transferred into each fresh tube (100000 cells/tube) and permeabilized with Saponin (1ml/tube), on ice for 10 minutes, preserving Ki-67 antigen. At the end of the incubation period with Saponin, cells were centrifuged at 1200 rpm for 5 minutes and the supernatant was removed. Cells were stained with the mouse monoclonal antibody Ki-67 FITC-conjugated (*BD Bioscience, San Diego, CA*). 20 µl of Ki-67 antibody were added to each tube and incubated in the dark for 30 minutes, at 4°C. As positive control it was used the Ki-67 isotype control (*BD Bioscience, San Diego, CA*) and as negative control the primary antibody was omitted and only a secondary FITC antibody was used. Then, cells were washed with FACS buffer (PBS with 2% FBS – 2 ml/tube), centrifuged at 1200 rpm for 5 minutes and re-suspended in 0,5ml of FACS buffer (PBS with 2% FBS). Finally, the cell samples were analyzed with FACS (*BD FACSCanto™- BD Bioscience, San Jose, CA*) and the data were analyzed using the BD FACSDiva™ software version 8.8.7 (*BD Bioscience, San Jose, CA*).

3.5 Cell count

An automated cell counter (*Scepter™ 2.0 Cell Counter, Millipore*), was used to count the total number of each cell type, after the three different treatments. Briefly, cells were detached with the Trypsin (0,025%)/EDTA (0,01%) solution (*Promocell, Heidelberg, Germany*) and centrifuged at 1000 rpm for 5 minutes. After removing the supernatant, pellet was re-suspended in the appropriate culture medium. Cell suspension (200 µl) was transferred into an eppendorf tube of 2ml, with a round base for Scepter cell count. Depending on the cell diameter, a specific sensor (40 µm or 60 µm) was attached to the Scepter, the plunger was depressed and the sensor was submerged into the sample; then the plunger was released drawing 50 µL of cell suspension through the cell sensing aperture. In this way, the Scepter cell counter provided the cell concentration, displaying an histogram as a function of cell diameter or volume on its screen. Using the Scepter 2.0 software, the test files were then uploaded from the device in order to perform data analysis to determine the final cell number.

3.6 Immunohistochemical analysis

At the end of the culture period, the different cell types were fixed on coverslips so to evaluate some cell markers through immunohistochemistry. The goal of fixation is to halt cells decomposition and freeze cellular proteins and subcellular structures in place. The cells were fixed by cross-linking, incubating them with 2 to 4% paraformaldehyde solution for 10 to 20 minutes at room temperature. At the end of the incubation period, the cells were carefully washed with PBS (*Phosphate Buffered Saline, Amresco*) to remove any fixation agent. Then the coverslips were allowed to dry at room temperature for 24 hours.

3.6.1 Collagen type I (Col I)

Immunohistochemical analysis for Collagen type I were performed on NHDF and HOBs. Collagen I is synthesized mainly by fibroblasts, osteoblasts, odontoblasts and chondroblasts. It attends a structural role in the extracellular matrix by providing mechanical support and resistance to tension. Immunohistochemistry was performed using the UltraVision Quanto Detection System Horseradish Peroxidase (*HRP; ThermoScientific, Bio-Optica, Milan, Italy*), followed by development with the chromogen substrate Diaminobenzidine (*DAB, Amresco, Prodotti Gianni, Milan, Italy*). Before adding the mouse monoclonal antibody, the cells were permeabilized with Triton 0,1% for 10 minutes and then incubated with blocking solution for 5 minutes. The primary antibody used was Col I (1:100, *Abcam, Cambridge*). To better visualize the positive reaction, the cells were counterstained with Carazzi's Hematoxylin, dehydrated and mounted with DPX, for light microscopy detection and quantification of Col I levels. Digitally fixed images of cells were analyzed using an image analyzer (*Image Pro-Plus 9.1.4, Milan, Italy*) and the Integrated Optical Density (IOD) was measured.

3.6.2 Osteopontin (OPN)

Immunohistochemical analysis for Osteopontin was performed on HOBs. OPN is a multifunctional 41 KDa protein, expressed by osteoblasts during cell proliferation and matrix mineralization. The UltraVision Quanto Detection System Horseradish Peroxidase (*HRP; ThermoScientific, Bio-Optica, Milan, Italy*), was used to perform the immunohistochemical analysis, followed by development with DAB. Before adding the mouse monoclonal antibody, the cells were permeabilized with Triton 0,1% for 10 minutes and then incubated with blocking solution for 5 minutes. The primary antibody used was Anti-Osteopontin (1:100, *Abcam, Cambridge*). To better

visualize the positive reaction, the cells were counterstained with Carazzi's Hematoxylin (*Bio-Optica, Milan, Italy*), dehydrated and mounted with DPX, for light microscopy detection and quantification of OPN levels. Digitally fixed images of cells were analyzed using an image analyzer (*Image Pro-Plus 9.1.4, Milan, Italy*) and the Integrated Optical Density was measured and quantified.

3.6.3 Vascular Endothelial Growth Factor (VEGF) and endothelial Nitric Oxide Synthase (eNOS).

Immunohistochemical analysis for VEGF and eNOS was performed on HUVEC. VEGF is a potent and essential angiogenic growth factor for vascular endothelial cells. eNOS plays crucial roles in regulating vascular tone, cellular proliferation, leukocyte adhesion and platelet aggregation. The UltraVision Quanto Detection System Horseradish Peroxidase (*HRP; ThermoScientific, Bio-Optica, Milan, Italy*), was used to perform the immunohistochemical analysis, followed by development with DAB. Cells were first permeabilized with Triton 0,1% for 10 minutes and then incubated with the blocking solution for 5 minutes. The primary antibodies used were VEGF (1:100, *Santa Cruz Biotechnology*) and NOS3 or eNOS (1:100, *Santa Cruz Biotechnology*). To better visualize the positive reaction, the cells were counterstained with Carazzi's Hematoxylin (*Bio-Optica, Milan, Italy*), dehydrated and mounted with DPX, for light microscopy detection and quantification of OPN levels. Digitally fixed images of cells were analyzed using an image analyzer (Image Pro-Plus 9.1.4, *Milan, Italy*) and the Integrated Optical Density was measured and quantified.

3.7 Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). Differences among groups were analyzed by a one-way analysis of variance (ANOVA test), using Bonferroni's multiple comparison test for post-hoc analysis. The level of significance was accepted at $*p < 0.05$. Origin v9.0 software was used for all statistical analyses performed.

4. RESULTS

4.1 FACS analysis and cell count

4.1.1 Cells treated with Sodium Orthosilicate

1) NHDF

FACS analysis for the quantification of ki-67 percentage, showed that there were no statistical differences between NHDF treated and not with Na_4SiO_4 0,5 and 1 mM. As shown in figure 9, in complete medium (FGM) the percentage of Ki-67 positive cells (64% ± 2,79) was quite similar to that of FGM + Na_4SiO_4 0,5 mM (67,6% ± 5,5) and FGM + Na_4SiO_4 1mM (68,2% ± 4,9). In fact there were no statistical differences among these treatments. Similar results were obtained also performing a simple cell count, using an automated cell counter (*Scepter™ 2.0 Cell Counter, Millipore*). The number of cells didn't vary significantly in NHDF treated with Na_4SiO_4 0,5 mM (3,80 X 10^4 ± 0,3) and Na_4SiO_4 1 mM (3,90 X 10^4 ± 0,3), compared with FGM alone (2,46 X 10^4 ± 0,2). Moreover, Na_4SiO_4 treatment didn't alter cell morphology. In fact, the cells showed a clear characteristic spindle-like morphology, appearing elongated and well spread on the plate surface.

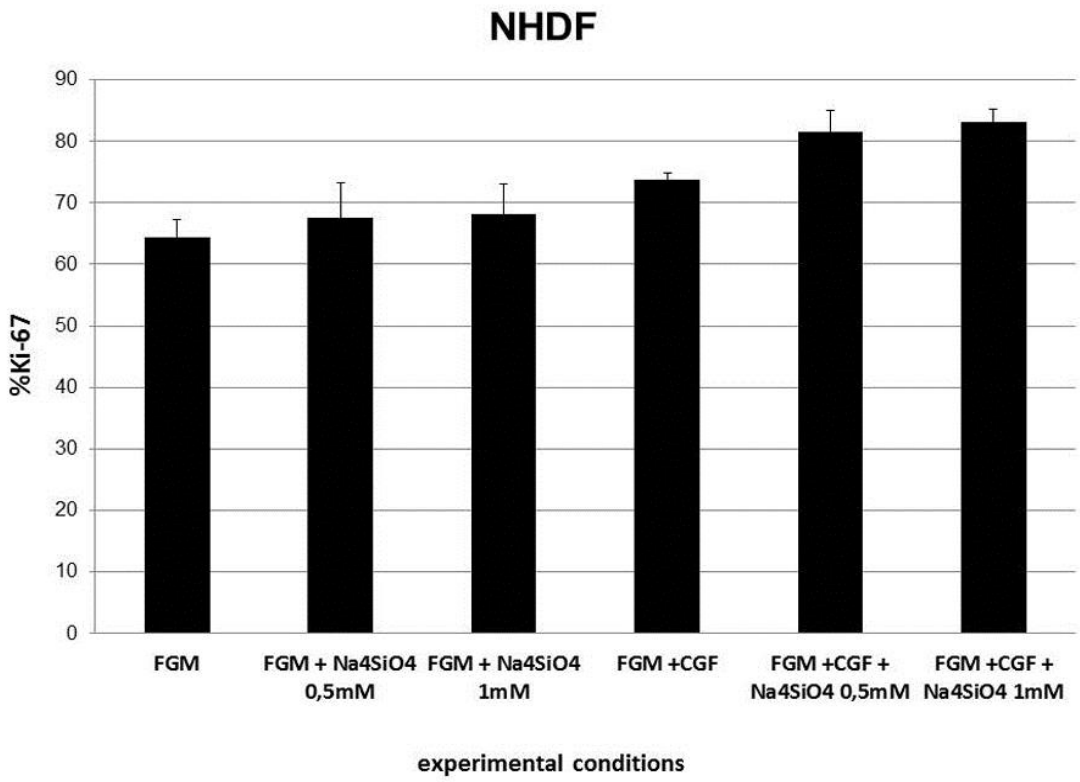

Figure 9: Ki-67 expression in NHDF. Graph comparing six experimental groups: A) FGM; B) FGM + Na_4SiO_4 0,5mM; C) FGM + Na_4SiO_4 1mM; D) FGM + CGF; E) FGM + CGF + Na_4SiO_4 0,5mM; E) FGM + CGF + Na_4SiO_4 1mM. The Ki-67 positive cells are reported as % ± SE.

2) HUVEC

FACS analysis for the quantification of ki-67 expression, showed that in complete medium alone (EGM), the percentage of Ki-67 positive cells was lower (67,3% ± 1,2) compared with EGM + Na_4SiO_4 0,5 mM (77,6% ± 0,5) and EGM + Na_4SiO_4 1 mM (70,7% ± 0,6), (Figure 10). In particular, treatment with EGM + Na_4SiO_4 0,5 mM, significantly increased the percentage of ki-67 positive cells, respect to EGM alone. Also treatment with EGM + Na_4SiO_4 1 mM showed a little increase in Ki-67 percentage but it was no statistically significant. The same data were obtained performing a simple cell count, using an automated cell counter (*Scepter™ 2.0 Cell*

Counter, Millipore). The number of cells resulted markedly increased after treatment with EGM + Na_4SiO_4 0,5 mM (3,80 x 10^5 ± 0,5) and EGM + Na_4SiO_4 1 mM (3,1 X 10^5 ± 0,5) respect to EGM alone (2,9 x 10^5 ± 0,2). Moreover, Na_4SiO_4 treatment didn't alter cell morphology. In fact, the cells showed their typical polygonal shape morphology appearing well attached on the plate surface.

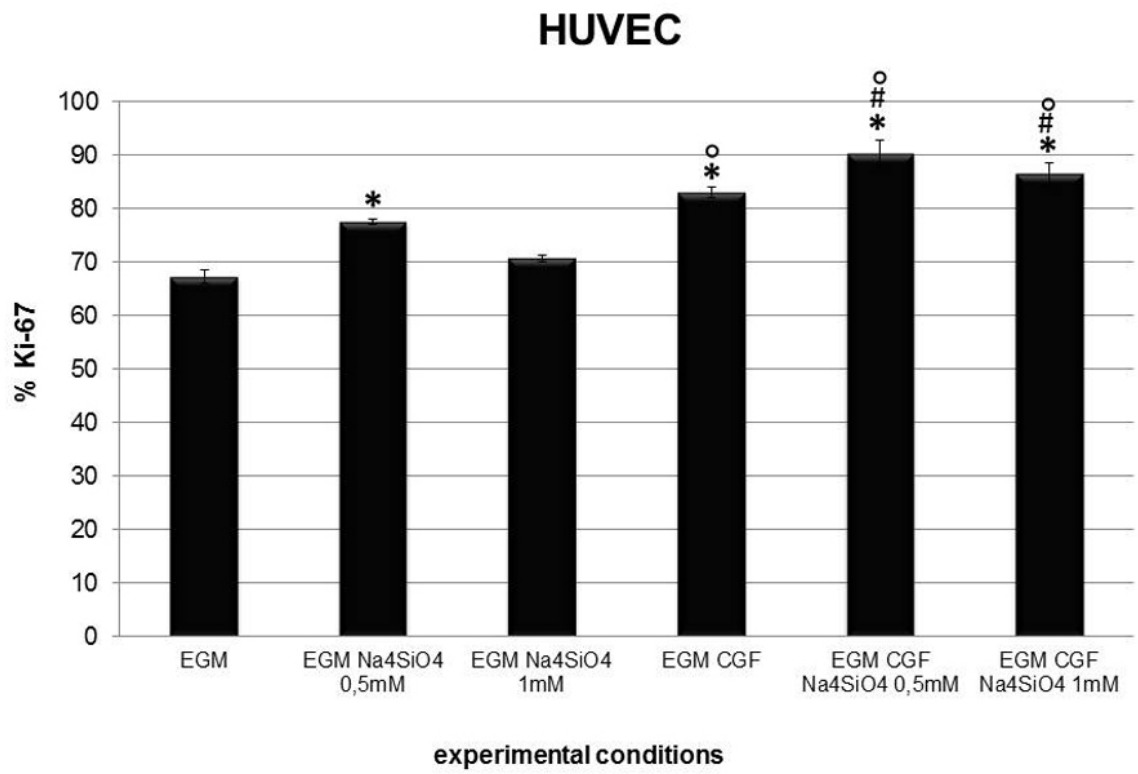

Figure 10: Ki-67 expression in HUVEC. Graph comparing six experimental groups: A) EGM; B) EGM + Na_4SiO_4 0,5mM; C) EGM + Na_4SiO_4 1mM; D) EGM + CGF; E) EGM + CGF + Na_4SiO_4 0,5mM; F) EGM + CGF + Na_4SiO_4 1mM. The Ki-67 positive cells are reported as % ± SE. *p<0,05 vs EGM; # p<0,05 vs EGM + Na_4SiO_4 0,5mM; °p<0,05 vs EGM + Na_4SiO_4 1mM.

3) HOBs

FACS analysis for the quantification of ki-67 percentage, showed that there were no statistical differences between HOBs treated and not with Na_4SiO_4 0,5 and 1 mM. As

shown in figure 11, in complete medium (OGM) the percentage of Ki-67 positive cells (48% ± 3,8) was similar to that of OGM + Na_4SiO_4 0,5 mM (50,4% ± 3,5) and OGM + Na_4SiO_4 1mM (52,2% ± 1,5). In fact there were no statistical differences among the different treatments. Similar results were obtained performing a simple cell count, using an automated cell counter (*Scepter™ 2.0 Cell Counter, Millipore*). In fact, even if the number of cells didn't vary significantly with addition of Na_4SiO_4 0,5 mM and 1mM, it was a little bit higher in OGM + Na_4SiO_4 0,5 mM (1,03 X 10^5 ± 0,3) and OGM + Na_4SiO_4 1 mM (1,31 X 10^5 ± 0,2), respect to OGM alone (9,64 X 10^4 ± 0,4). Moreover, treatment with Na_4SiO_4 didn't alter change cell morphology. In fact, the cells showed their typical polygonal and flattened shape morphology and appeared well attached on the plate surface.

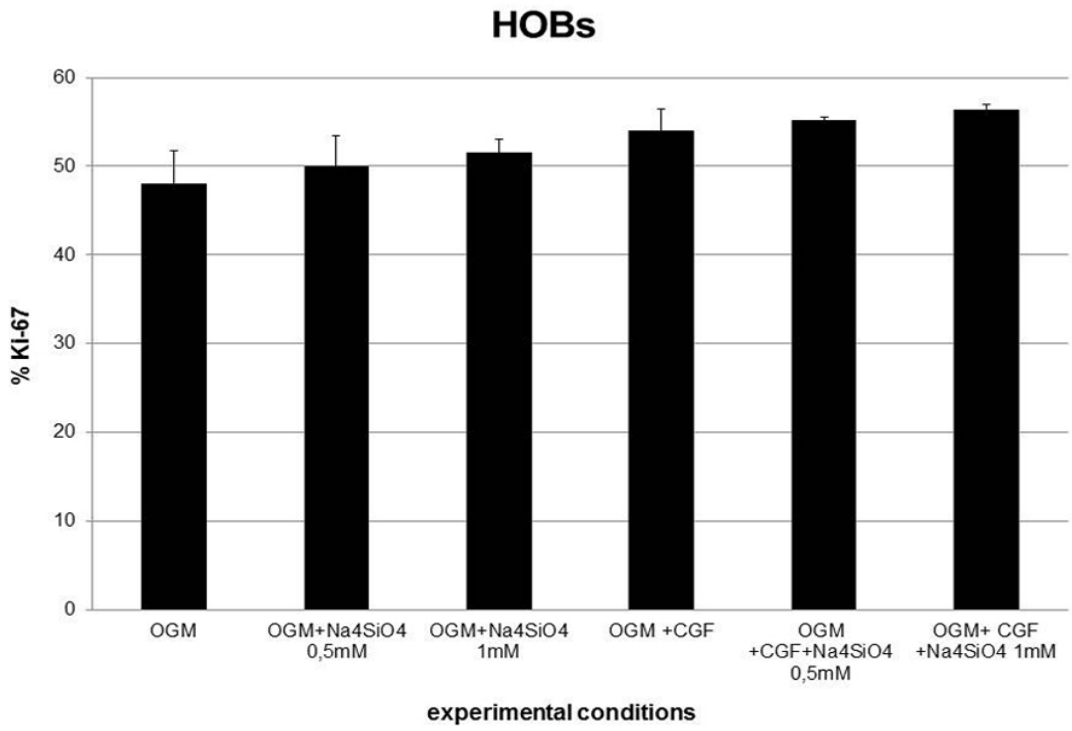

Figure 11: Ki-67 expression in HOBs. Graph comparing six experimental groups: A) OGM; B) OGM + Na_4SiO_4 0,5mM; C) OGM + Na_4SiO_4 1mM; D) OGM + CGF; E) OGM + CGF + Na_4SiO_4 0,5mM; F) OGM + CGF + Na_4SiO_4 1mM. The Ki-67 positive cells are reported as % ± SE.

4.1.2 Cells treated with CGF

1) NHDF

As reported in figure 12, FACS analysis for the quantification of ki-67 percentage, showed that in basal medium (FBM), free of serum and growth factors, the percentage of Ki-67 positive cells was lower (18,68% ± 1,8) compared with the other experimental conditions. Moreover, the cells did not show a clear spindle-like morphology, appearing short and not well spread on the plate surface. In complete medium (FGM), the percentage of Ki-67 positive cells was significantly higher (61,98% ± 6,35) respect to FBM and the cells showed a clear characteristic spindle-like morphology, appearing elongated and well spread on the plate surface. In basal medium with CGF (FBM + CGF), the percentage of Ki-67 positive cells significantly increased (51,51% ± 7,12) compared with FBM alone and the cells showed a clear characteristic spindle-like morphology, appearing elongated but not well spread on the plate surface as in FBM. In complete medium with CGF (FGM + CGF), the percentage of Ki-67 positive cells was significantly higher (75,9% ± 4,13) respect to both FBM alone and FBM + CGF. Moreover the cells showed a clear characteristic spindle-like morphology, appearing well elongated, larger and well spread on the plate surface compared with the other culture conditions.

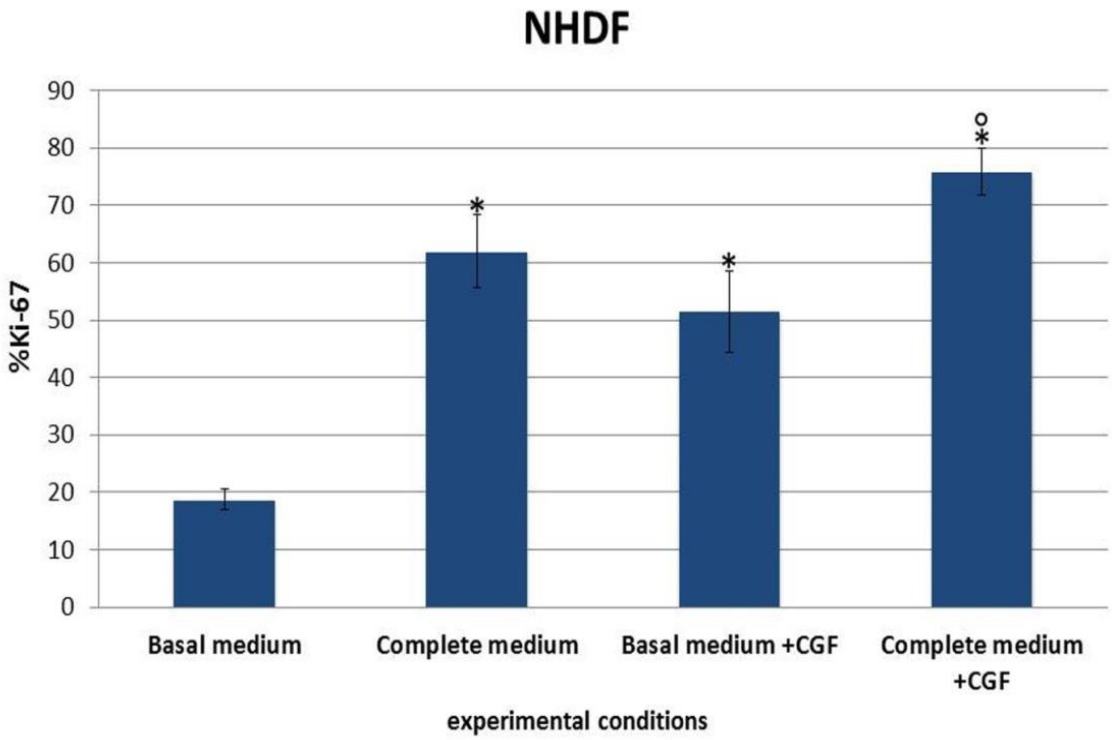

Figure 12: Effect of CGF on NHDF. Graph comparing four experimental groups: A) Basal medium (BM); B) Complete medium (CM); C) Basal medium + CGF (BM+CGF); D) Complete medium + CGF (CM + CGF). The Ki-67 positive cells are reported as % ± SE. *P<0,05 vs BM; °P<0,05 vs BM+CGF.

2) HUVEC

As reported in figure 13, FACS analysis for the quantification of ki-67 percentage showed that in basal medium (EBM), free of serum and growth factors, the percentage of Ki-67 positive cells was significantly lower (5,05% ± 0,19) compared with the other experimental conditions. This because HUVEC are extremely delicate and their growth is strongly influenced by culture conditions. In fact the cells showed a round shape morphology and appeared not well attached on the plate surface. In complete medium (EGM), the percentage of Ki-67 positive cells was significantly

higher (26,03% ± 2,79) compared with EBM alone and the cells showed their typical polygonal shape morphology, appearing well attached on the plate surface. Also in basal medium with CGF (EBM + CGF), the percentage of Ki-67 positive cells markedly increased (26,94% ± 1,96) compared with the EBM alone and the cells showed a more defined polygonal shape morphology appearing larger and well attached on the plate surface compared with EBM and EGM alone. In EGM + CGF, the percentage of Ki-67 positive cells markedly increased (38,62% ± 4,03) compared with all the other culture conditions. The cells showed a more defined polygonal shape morphology appearing larger and well attached on the plate surface compared all the other culture conditions.

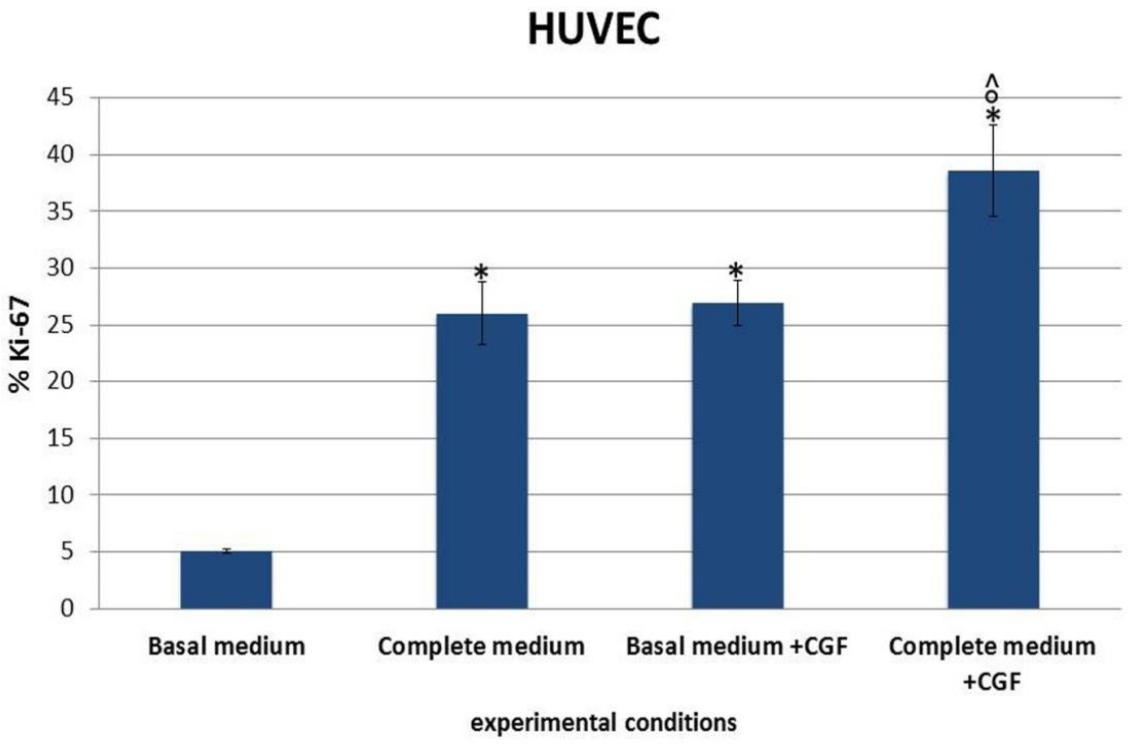

Figure 13: Effect of CGF on HUVEC. Graph comparing four experimental groups: A) Basal medium (BM); B) Complete medium (CM); C) Basal medium + CGF (BM+CGF); D) Complete medium + CGF (CM + CGF). The Ki-67 positive cells are reported as % ± SE. *P<0,05 vs BM; °P<0,05 vs BM+CGF; ^P<0,05 vs CM.

3) HOBs

As shown in figure 14, FACS analysis for the quantification of ki-67 percentage showed that in basal medium (OBM), free of serum and growth factors, the cells showed the lowest percentage of Ki-67 (15,07% ± 0,39), compared with the other experimental conditions. The cells showed their typical polygonal and flattened shape morphology and appeared well attached on the plate surface. In complete medium (OGM), the percentage of Ki-67 positive cells significantly increased (35,31% ± 1,21) compared with the basal medium and the cells showed a more elongated polygonal and flattened shape morphology with the presence of extensions or very thin filopodia compared with OBM. In basal medium with CGF (OBM + CGF), the percentage of Ki-67 positive cells markedly increased (32,3% ± 2,46), resulting statistically different from OBM alone. The cells showed a more elongated, larger polygonal and flattened shape morphology with the presence of more extensions or very thin filopodia respect to OBM and OGM. In complete medium with CGF (OGM + CGF), the percentage of Ki-67 positive cells markedly increased (38,13% ± 2,72) compared with OBM alone. The cells showed a more elongated, larger polygonal and flattened shape morphology with the presence of more extensions or very thin filopodia compared with OBM and OGM.

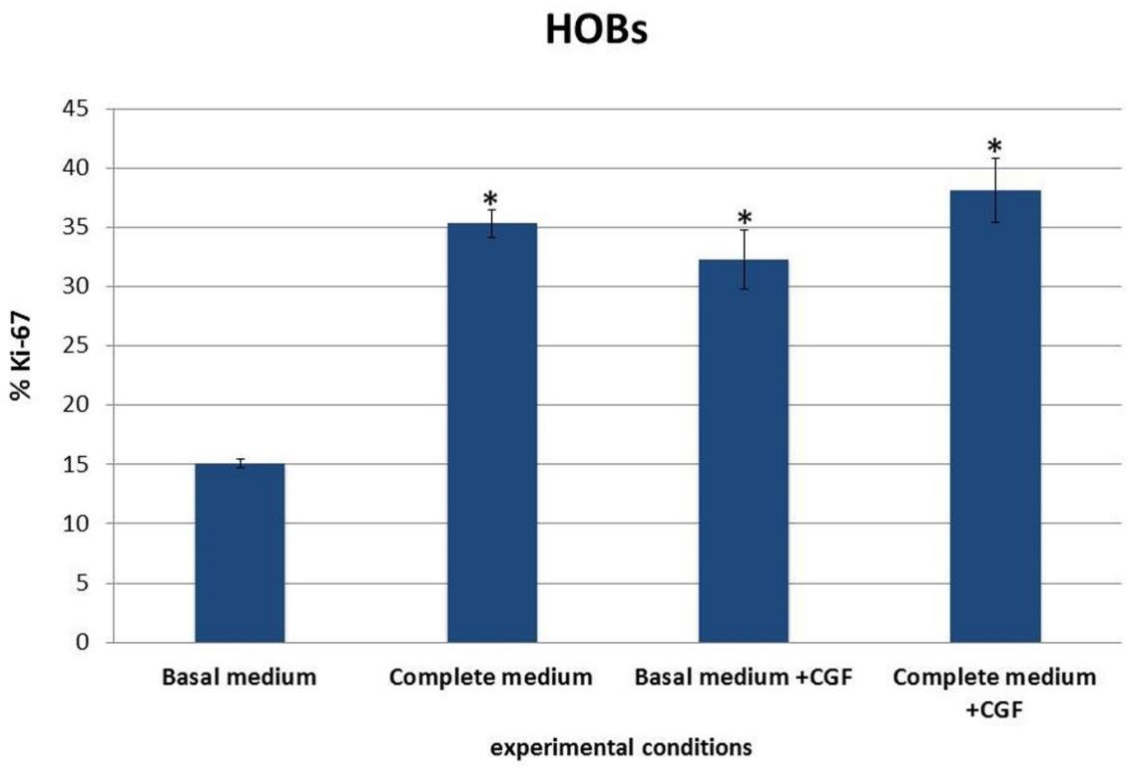

Figure 14: Effect of CGF on HOBs. Graph comparing four experimental groups: A) Basal medium (BM); B) Complete medium (CM); C) Basal medium + CGF (BM+CGF); D) Complete medium + CGF (CM + CGF). The Ki-67 positive cells are reported as % ± SE. *P<0,05 vs BM.

4.1.3 Cells treated with Sodium Orthosilicate and CGF

1) NHDF

FACS analysis for the quantification of ki-67 percentage, showed that treatment with Sodium Orthosilicate and CGF did not significantly influence NHDF growth and proliferation (Figure 9, page 46), even if the percentage of Ki-67 positive cells was higher in NHDF treated with both CGF and Na_4SiO_4 0,5 mM and 1 mM, respect to FGM supplemented and not with Sodium Orthosilicate. In FGM + CGF, the

percentage of Ki-67 positive cells (73,8% ± 2,79) was slightly lower compared with FGM + CGF + Na_4SiO_4 0,5 mM (81,6% ± 3,5) and FGM + CGF + Na_4SiO_4 1 mM (83,1% ± 2,03). However this difference was not statistically significant. In FGM + CGF + Na_4SiO_4 0,5 mM and FGM + CGF + Na_4SiO_4 1 mM, the percentage of Ki-67 positive cells was very similar but it was higher respect to FGM + CGF and also compared with FGM supplemented and not with Na_4SiO_4 0,5 and 1 mM. Similar results were obtained performing a simple cell count, using an automated cell counter (Scepter™ 2.0 Cell Counter, Millipore). In fact, even if the number of cells did not vary significantly with addition of Na_4SiO_4 0,5 mM and 1 mM, it was higher in FGM + CGF + Na_4SiO_4 0,5 mM (5,22 x 10^4 ± 0,4) and FGM + Na_4SiO_4 1 mM (6,23 x 10^4 ± 0,4) respect to FGM + CGF (3,27 x 10^4 ± 0,3). Moreover, treatment with Na_4SiO_4 did not alter cell morphology. In fact, the cells showed their typical polygonal and flattened shape morphology and appeared well attached on the plate surface.

2) HUVEC

FACS analysis for the quantification of ki-67 percentage, showed that treatment with Na_4SiO_4 and CGF significantly influenced HUVEC growth and proliferation, as reported in figure 10 (page 47). In fact, in EGM + CGF, the percentage of Ki-67 positive cells (83% ± 1) markedly increased respect to EGM alone (67,3% ± 1,2) and EGM + Na_4SiO_4 1 mM (70,7 ± 0,6). In EGM + CGF + Na_4SiO_4 0,5 mM, the percentage of Ki-67 positive cells (90,2% ± 2,7) markedly increased respect to EGM alone, EGM + Na_4SiO_4 0,5 mM (77,6% ± 0,5) and EGM + Na_4SiO_4 1 mM (70,7% ± 0,6). Similarly to EGM + CGF + Na_4SiO_4 0,5 mM, also in EGM + CGF + Na_4SiO_4 1 mM, the percentage of Ki-67 positive cells (86,4% ± 2) markedly increased respect to EGM alone, EGM + Na_4SiO_4 0,5 mM and EGM + Na_4SiO_4 1 mM. Moreover, comparing the percentage of Ki-67 positive cells in EGM + CGF with EGM +

Na_4SiO_4, it was statistically different respect to EGM + Na_4SiO_4 1 mM. Similar results were obtained counting the number of cells (EGM + CGF: 4,20 x 10^5 ± 0,2; EGM + CGF + Na_4SiO_4 0,5 mM: 4,95 x 10^5 ± 0,6; EGM + CGF + Na_4SiO_4 1 mM: 4,95 x 10^5 ± 0,5).

3) HOBs

FACS analysis for the quantification of ki-67 percentage, showed that there were no statistical differences after cell treatment with Sodium Orthosilicate and CGF (Figure 11, page 48). In OGM + CGF, the percentage of Ki-67 positive cells (54% ± 2,4) was similar compared with FGM + CGF + Na_4SiO_4 0,5 mM (55,2 ± 0,3) and FGM + CGF + Na_4SiO_4 1 mM (56,4 ± 0,6), as reported in figure 10. Also the number of cells was similar among the different treatments (OGM + CGF: 1,70 x 10^5 ± 0,2; OGM + CGF + Na_4SiO_4 0,5 mM: 1,72 x 10^5 ± 0,1; OGM + CGF + Na_4SiO_4 1 mM: 1,75 x 10^5 ± 0,1). However, comparing these data with those obtained using only Na_4SiO_4 0,5 mM and 1 mM without CGF, the percentage of Ki-67 positive cells progressively increased using CGF togheter with Na_4SiO_4, even if this increment was not statistically significant.

4.2 Immunohistochemistry on fixed cells

4.2.1 Collagen type I (Col I)

NHDF

A positive immunostaining for Col I was observed in NHDF, in matrix and intracellular compartment and the intensity of the reaction progressively increased in cells treated with CGF supplemented and not with Na_4SiO_4 (Figures 15-16). In fact, in FGM immunopositivity was lower (20,8 ± 0,2), compared with the other experimental conditions. In FGM + Na_4SiO_4 0,5 mM immunopositivity (21,6 ± 0,4) was similar to FGM but lower than FGM + Na_4SiO_4 1 mM (23,3 ± 0,4). Immunopositivity for Col I, progressively increased in FGM + CGF (26,11 ± 1,6), FGM + CGF + Na_4SiO_4 0,5 mM (27,7 ± 0,6) and FGM + CGF + Na_4SiO_4 1 mM (29,1 ± 1,2), being statistically significant compared with FGM, FGM + Na_4SiO_4 0,5 mM and FGM + Na_4SiO_4 1 mM.

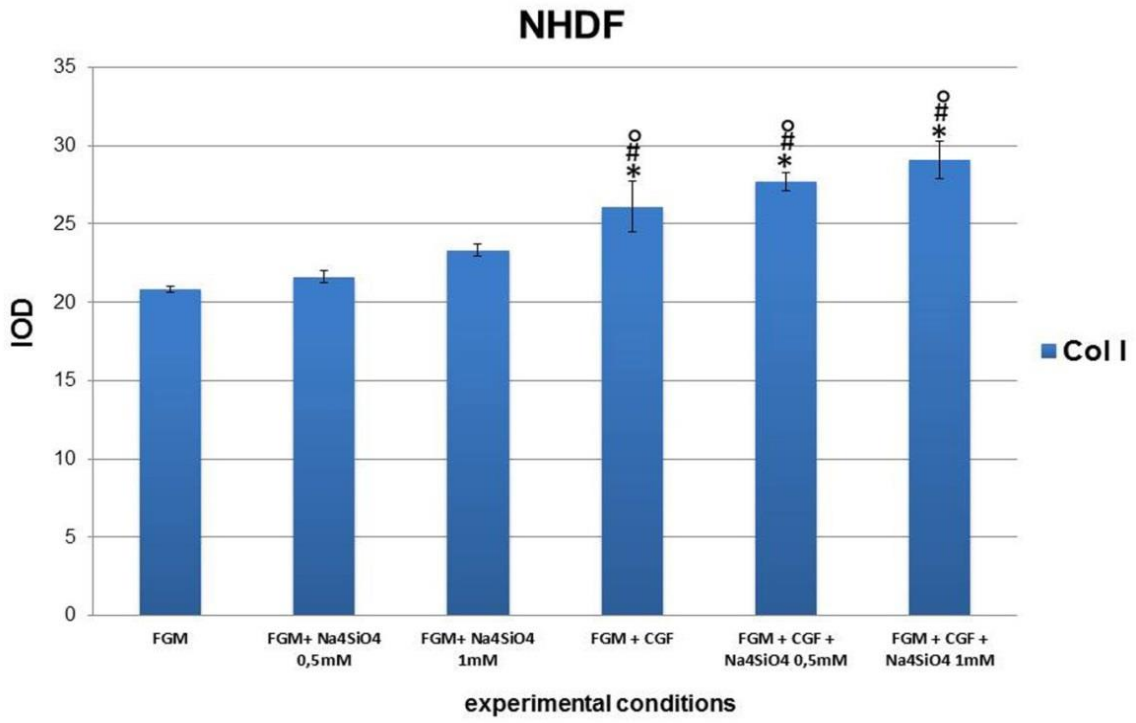

Figure 15: Integrated Optical Density analysis (IOD) after immunohistochemistry for Collagen type 1 in NHDF. Graph comparing six experimental groups: A) FGM; B) FGM + Na_4SiO_4 0,5mM; C) FGM + Na_4SiO_4 1mM; D) FGM + CGF; E) FGM + CGF + Na_4SiO_4 0,5mM; E) FGM + CGF + Na_4SiO_4 1mM. Data are expressed as means ± SEM.

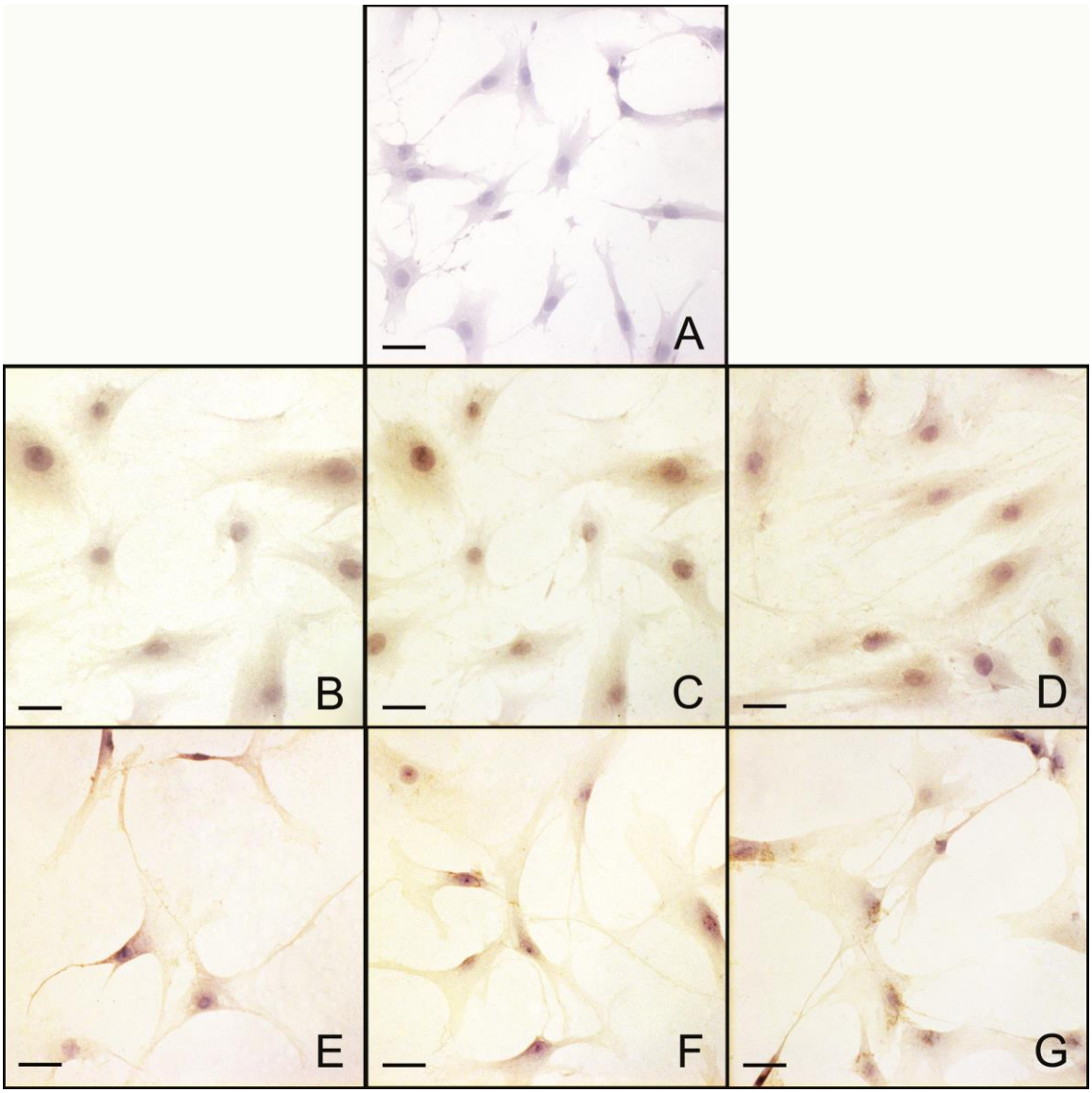

Figure 16: (A-G) Immunohistochemistry analysis (20X) for Collagen type 1 on NHDF comparing different experimental groups: A) Negative Control Group; B) Complete medium (FGM); C) FGM + Na_4SiO_4 0,5mM; D) FGM + Na_4SiO_4 1mM; E) FGM + CGF; F) FGM + CGF + Na_4SiO_4 0,5 mM; G) FGM + CGF + Na_4SiO_4 1mM (Bar=40µm).

HOBs

A positive immunostaining for Col I was found also around osteoblasts. The intensity of the reaction increased progressively among the different treatments (Figures 17-18). In fact, in OGM, immunopositivity for Col I was lower (10,01 ± 0,4), compared with the other experimental conditions. In OGM + Na_4SiO_4 0,5 mM immunopositivity was slightly higher (14,96 ± 0,6) compared with OGM alone but lower respect to the other treatments. In OGM + Na_4SiO_4 1 mM, there was a small increase in collagen I immunopositivity (16,65 ± 0,3) respect to OGM + Na_4SiO_4 0,5 mM and OGM but it was lower compared with the other experimental conditions. In hobs treated with OGM + CGF, Col I immunopositivity markedly increased (21,04 ± 0,4) compared with OGM, OGM + Na_4SiO_4 0,5 mM and OGM + Na_4SiO_4 1 mM but it was lower respect to OGM + CGF + Na_4SiO_4 0,5 mM (24,54 ± 0,7) and OGM + CGF + Na_4SiO_4 1 mM (26,16 ± 0,5). Both in OGM + CGF + Na_4SiO_4 0,5 mM and OGM + CGF + Na_4SiO_4 1 mM, immunopositivity greatly increased, being statistically significant respect to all the other treatments.

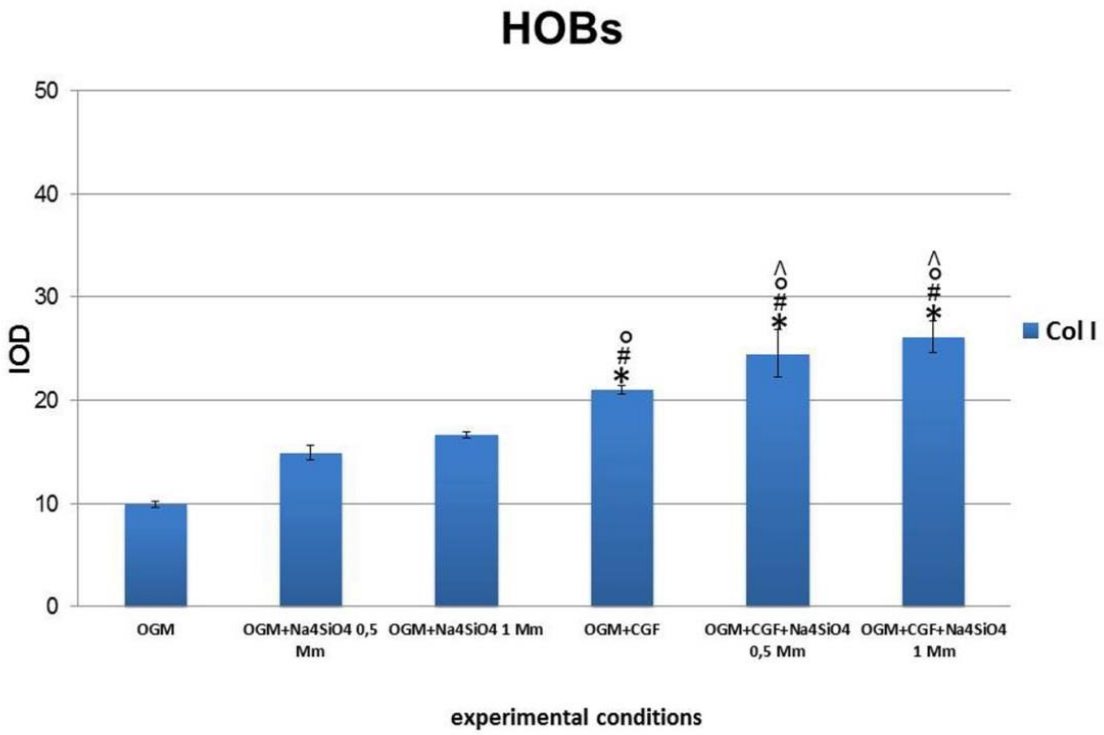

Figure 17: Integrated Optical Density analysis (IOD) after immunohistochemistry for Collagen type 1 in HOBs. Graph comparing six experimental groups: A) OGM; B) OGM + Na_4SiO_4 0,5mM; C) OGM + Na_4SiO_4 1mM; D) OGM + CGF; E) OGM + CGF + Na_4SiO_4 0,5mM; F) OGM + CGF + Na_4SiO_4 1mM. Data are expressed as means ± SEM. *p<0,05 vs OGM; # p<0,05 vs OGM + Na_4SiO_4 0,5mM; °p<0,05 vs OGM + Na_4SiO_4 1mM; Λ p<0,05 vs OGM + CGF.

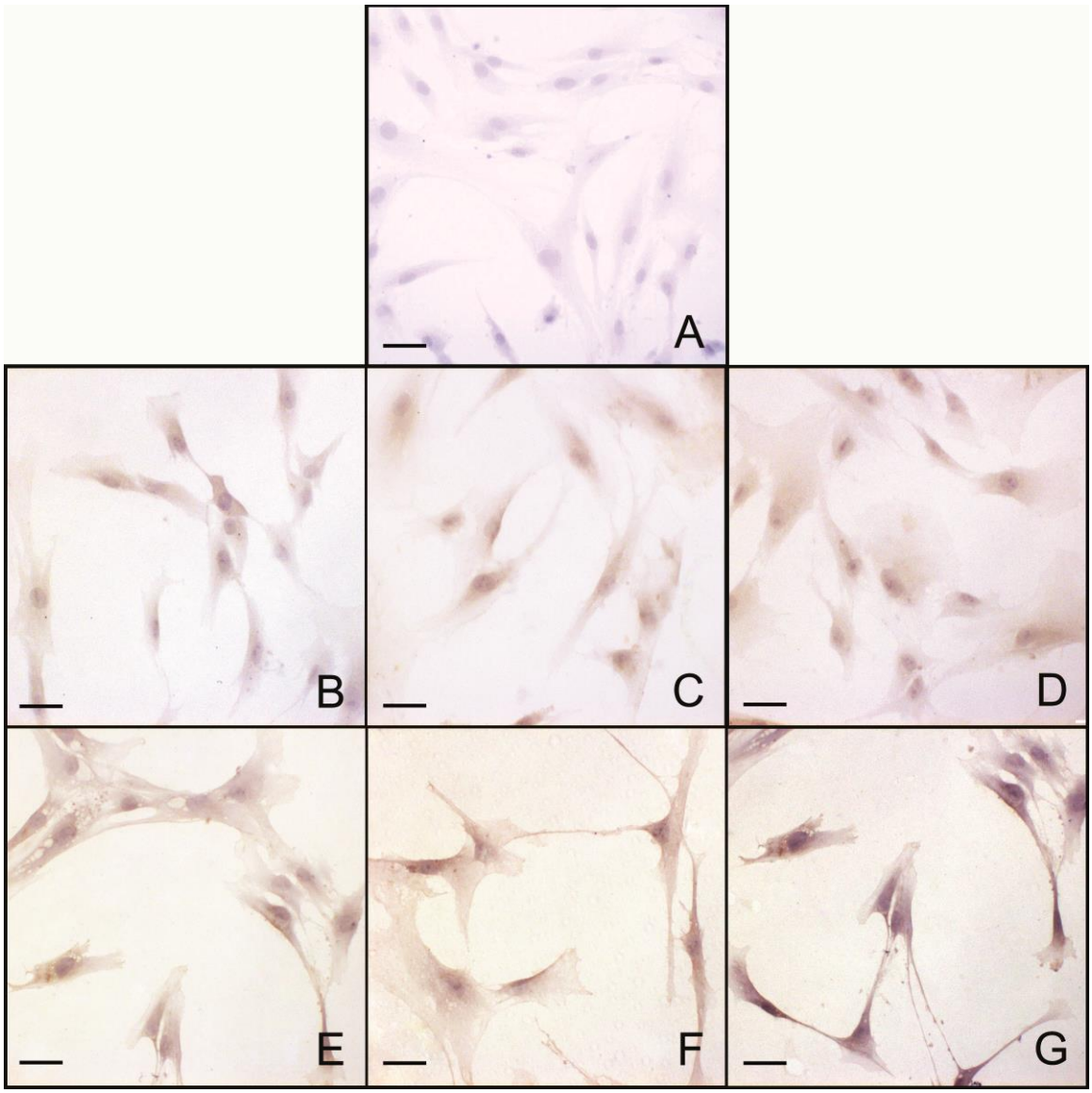

Figure 18: (A-G) Immunohistochemistry analysis (20X) for Collagen type 1 on HOBs comparing different experimental groups: A) Negative Control Group; B) Complete medium (OGM); C) OGM + Na_4SiO_4 0,5mM; D) OGM + Na_4SiO_4 1mM; E) OGM + CGF; F) OGM + CGF + Na_4SiO_4 0,5 mM; G) OGM + CGF + Na_4SiO_4 1mM (Bar=40µm).

4.2.2 Vascular Endothelial Growth Factor (VEGF)

HUVEC

A positive immunostaining for VEGF was observed in endothelial cells (Figures 19-20) and immunopositivity significantly increased in cells treated with CGF and Na_4SiO_4 0,5mM and 1 mM. In EGM + Na_4SiO_4 0,5 mM (35,15 ± 0,4) and EGM + Na_4SiO_4 1 mM (36,14 ± 0,3), VEGF immunopositivity was quite the same and it was statistically significant compared with EGM alone (28,52 ± 0,5), but lower respect to the other treatments. In EGM + CGF, VEGF immunopositivity markedly increased (40,8 ± 0,4), being statistically significant respect to EGM, EGM + Na_4SiO_4 0,5 mM and EGM + Na_4SiO_4 1 mM, but lower than EGM + CGF + Na_4SiO_4 0,5 mM and EGM + CGF + Na_4SiO_4 1 mM. EGM + CGF + Na_4SiO_4 0,5 mM (46,84 ± 0,8) and EGM + CGF + Na_4SiO_4 1mM (47,5 ± 1,2) showed a similar immunopositivity which was significantly higher compared with the other treatments but there was no statistical difference between this two treatments.

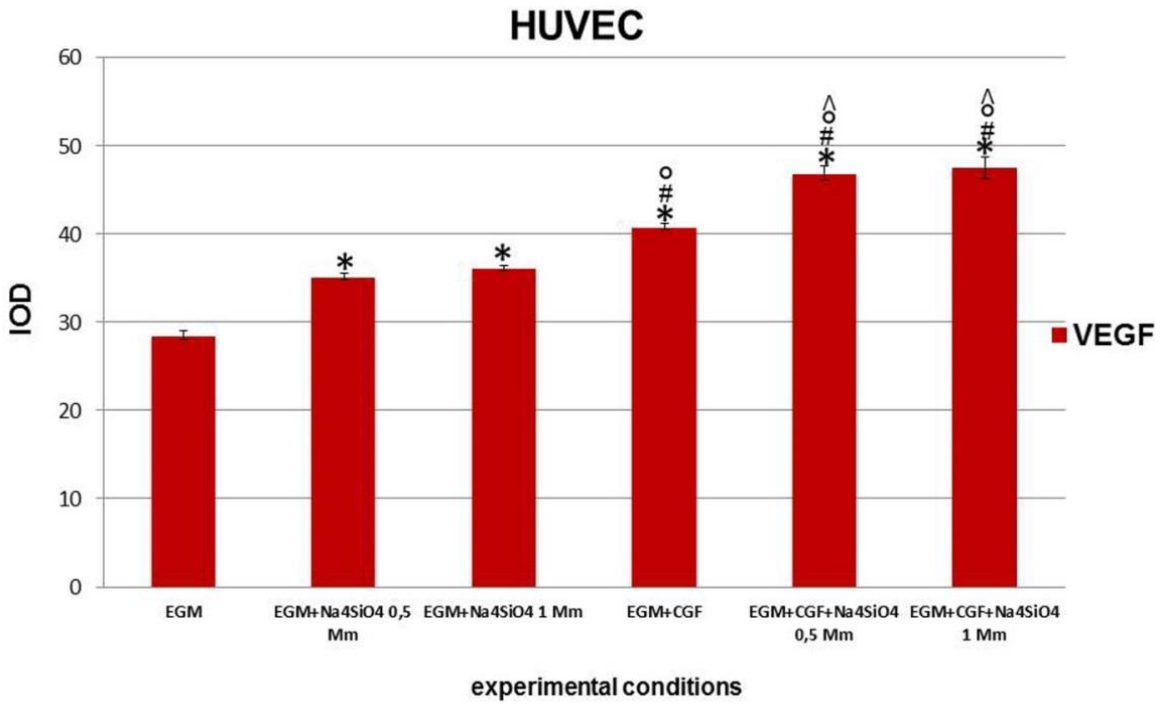

Figure 19: Integrated Optical Density analysis (IOD) after immunohistochemistry for VEGF on HUVEC. Graph comparing six experimental groups: A) EGM; B) EGM + Na_4SiO_4 0,5mM; C) EGM + Na_4SiO_4 1mM; D) EGM + CGF; E) EGM + CGF + Na_4SiO_4 0,5mM; F) EGM + CGF + Na_4SiO_4 1mM. Data are expressed as means ± SEM. *p<0,05 vs EGM; # p<0,05 vs EGM + Na_4SiO_4 0,5mM; °p<0,05 vs EGM + Na_4SiO_4 1mM; Λ p<0,05 vs EGM + CGF.

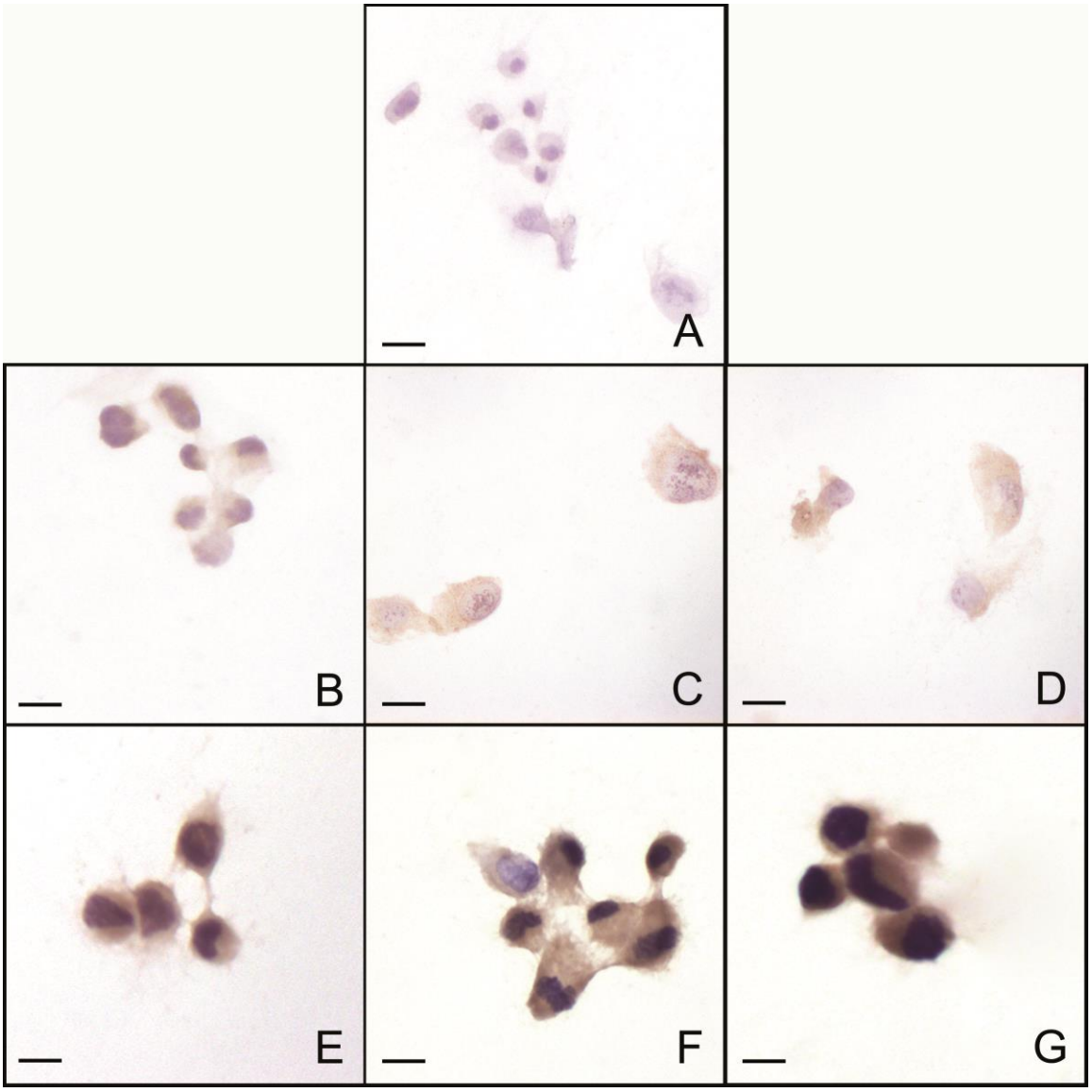

Figure 20: (A-G) Immunohistochemistry analysis (40X) for VEGF on HUVEC comparing different experimental groups: A) Negative Control Group; B) Complete medium (EGM); C) EGM + Na_4SiO_4 0,5mM; D) EGM + Na_4SiO_4 1mM; E) EGM + CGF; F) EGM + CGF + Na_4SiO_4 0,5 mM; G) EGM + CGF + Na_4SiO_4 1mM (Bar=60µm).

4.2.3 endothelial Nitric Oxide Synthase (eNOS)

A positive immunostaining for eNOS was observed in endothelial cells (Figures 21-22) and similarly to VEGF, immunopositivity significantly increased in cells treated with CGF and Na_4SiO_4 0,5mM and 1 mM. In EGM + Na_4SiO_4 0,5 mM (34,75 ± 0,7) and EGM + Na_4SiO_4 1 mM (35,86 ± 0,6), immunopositivity was quite the same and it was statistically significant compared with EGM alone (27,85 ± 0,5), but lower respect to the other treatments. In EGM + CGF, eNOS immunopositivity markedly increased (40,6 ± 0,8), being statistically significant respect to EGM, EGM + Na_4SiO_4 0,5 mM and EGM + Na_4SiO_4 1 mM, but lower than EGM + CGF + Na_4SiO_4 0,5 mM and EGM + CGF + Na_4SiO_4 1 mM. EGM + CGF + Na_4SiO_4 0,5 mM (47,43 ± 1,2) and EGM + CGF + Na_4SiO_4 1mM (48,64 ± 1,1) showed a similar immunopositivity which was significantly higher compared with the other treatments but there was no statistical differences between this two treatments.

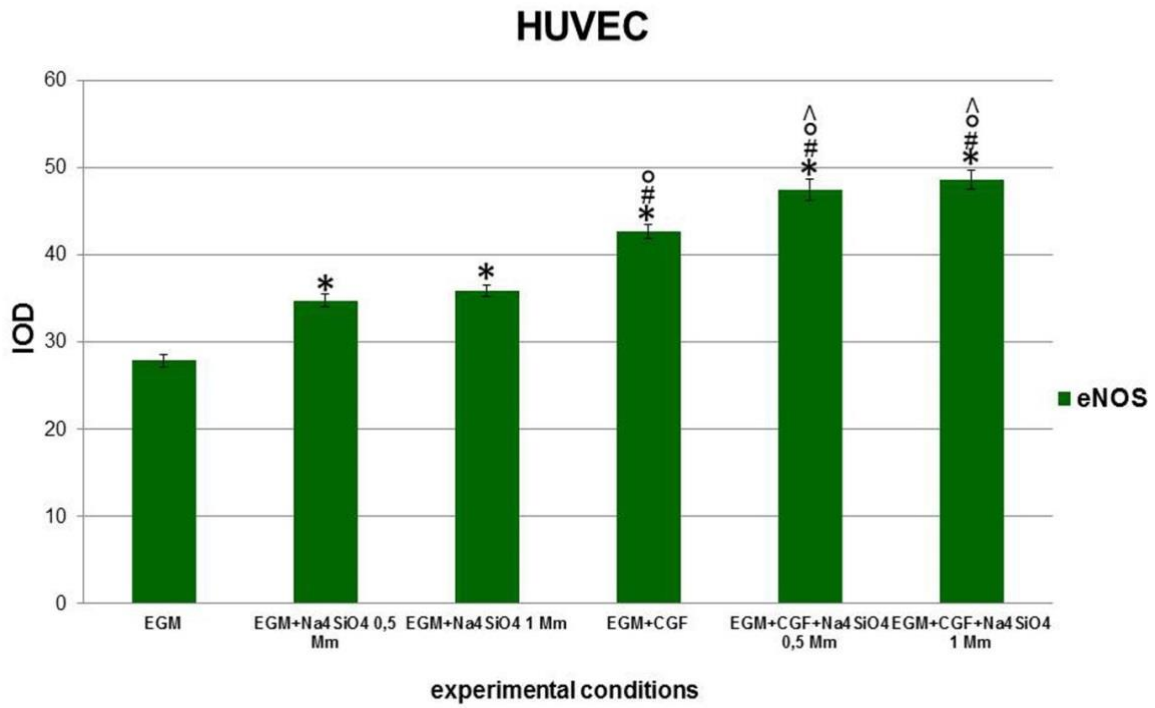

Figure 21: Integrated Optical Density analysis (IOD) after immunohistochemistry for eNOS on HUVEC. Graph comparing six experimental groups: A) EGM; B) EGM + Na_4SiO_4 0,5mM; C) EGM + Na_4SiO_4 1mM; D) EGM + CGF; E) EGM + CGF + Na_4SiO_4 0,5mM; F) EGM + CGF + Na_4SiO_4 1mM. Data are expressed as means ± SEM. *p<0,05 vs EGM; # p<0,05 vs EGM + Na_4SiO_4 0,5mM; °p<0,05 vs EGM + Na_4SiO_4 1mM; Λ p<0,05 vs EGM + CGF.

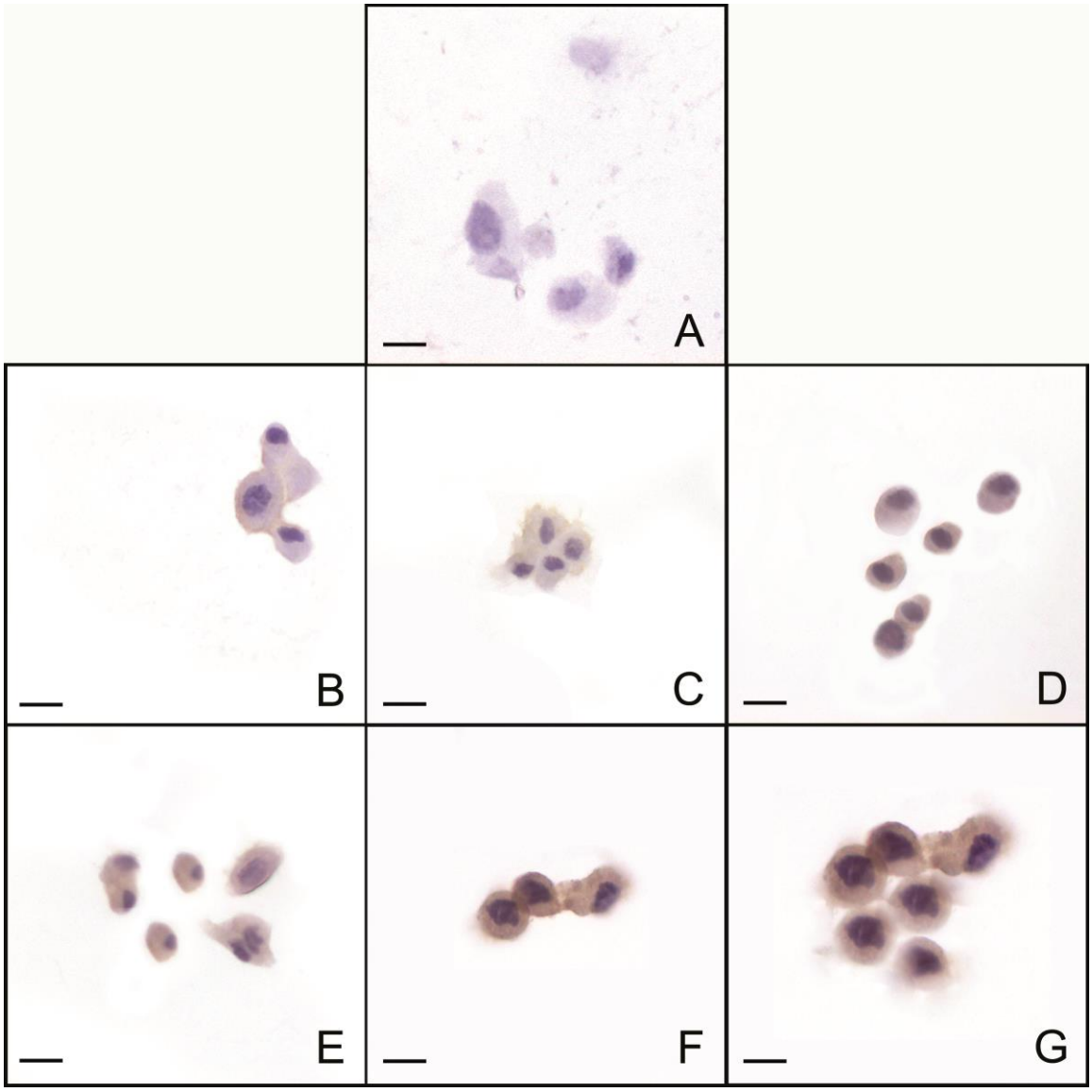

Figure 22: (A-G) Immunohistochemistry analysis (40X) for eNOS on HUVEC comparing different experimental groups: A) Negative Control Group; B) Complete medium (EGM); C) EGM + Na_4SiO_4 0,5mM; D) EGM + Na_4SiO_4 1mM; E) EGM + CGF; F) EGM + CGF + Na_4SiO_4 0,5 mM; G) EGM + CGF + Na_4SiO_4 1mM (Bar=60µm).

4.2.4 Osteopontin (OPN)

OPN immunopositivity was observed in osteoblast cells and it showed a similar trend to Col I, progressively increasing among the different treatments (Figures 23-24). In OGM + Na_4SiO_4 0,5 mM (19,45 ± 0,5) and OGM + Na_4SiO_4 1 mM (20,23 ± 0,9), OPN immunopositivity was quite the same and it was statistically significant compared with OGM alone (11,65 ± 0,3) but lower compared with CGF supplemented and not with Na_4SiO_4. In OGM + CGF immunopositivity markedly increased (23,41 ± 0,9), being statistically different from OGM. OGM + CGF + Na_4SiO_4 0,5 mM (25,97 ± 0,6) and OGM + CGF + Na_4SiO_4 1 mM (26,05 ± 0,6), showed a very similar amount in OPN (even if it was slightly higher in CGF + Na_4SiO_4 1 mM) and so there were not statistically differences between these two treatments which were statistically significant respect to the other treatments, except to OGM + CGF.

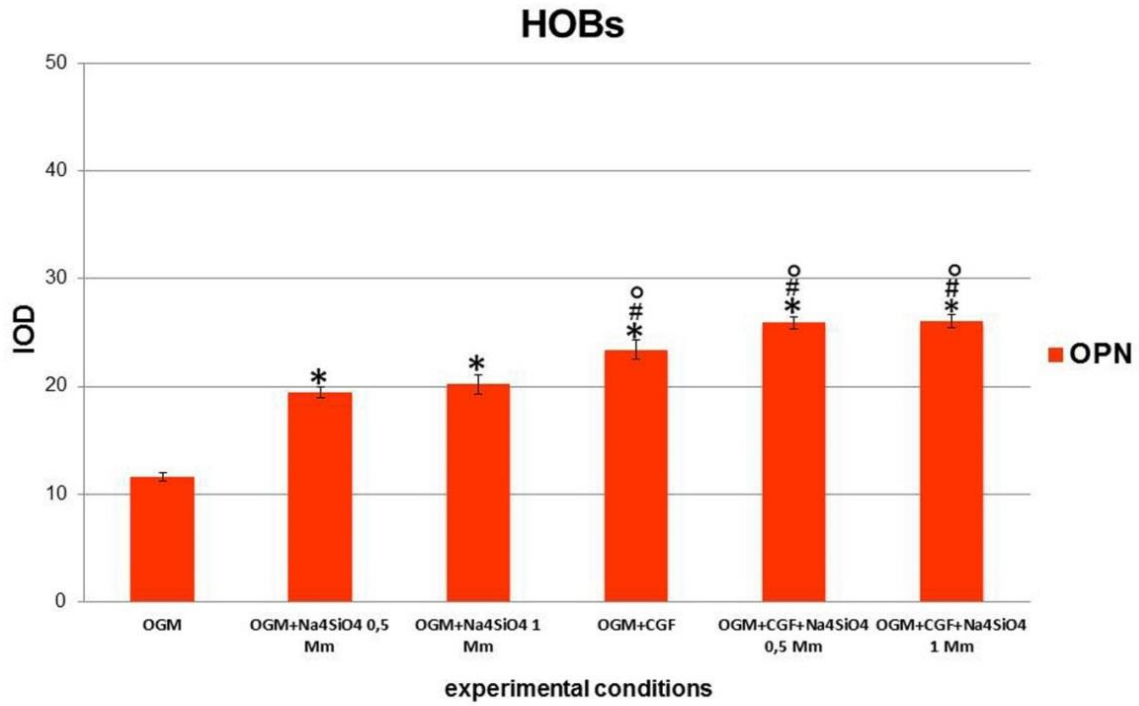

Figure 23: Integrated Optical Density analysis (IOD) after immunohistochemistry for Osteopontin in HOBs. Graph comparing six experimental groups: A) OGM; B) OGM + Na_4SiO_4 0,5mM; C) OGM + Na_4SiO_4 1mM; D) OGM + CGF; E) OGM + CGF + Na_4SiO_4 0,5mM; F) OGM + CGF + Na_4SiO_4 1mM. Data are expressed as means ± SEM. *p<0,05 vs OGM; # p<0,05 vs OGM + Na_4SiO_4 0,5mM; °p<0,05 vs OGM + Na_4SiO_4 1mM.

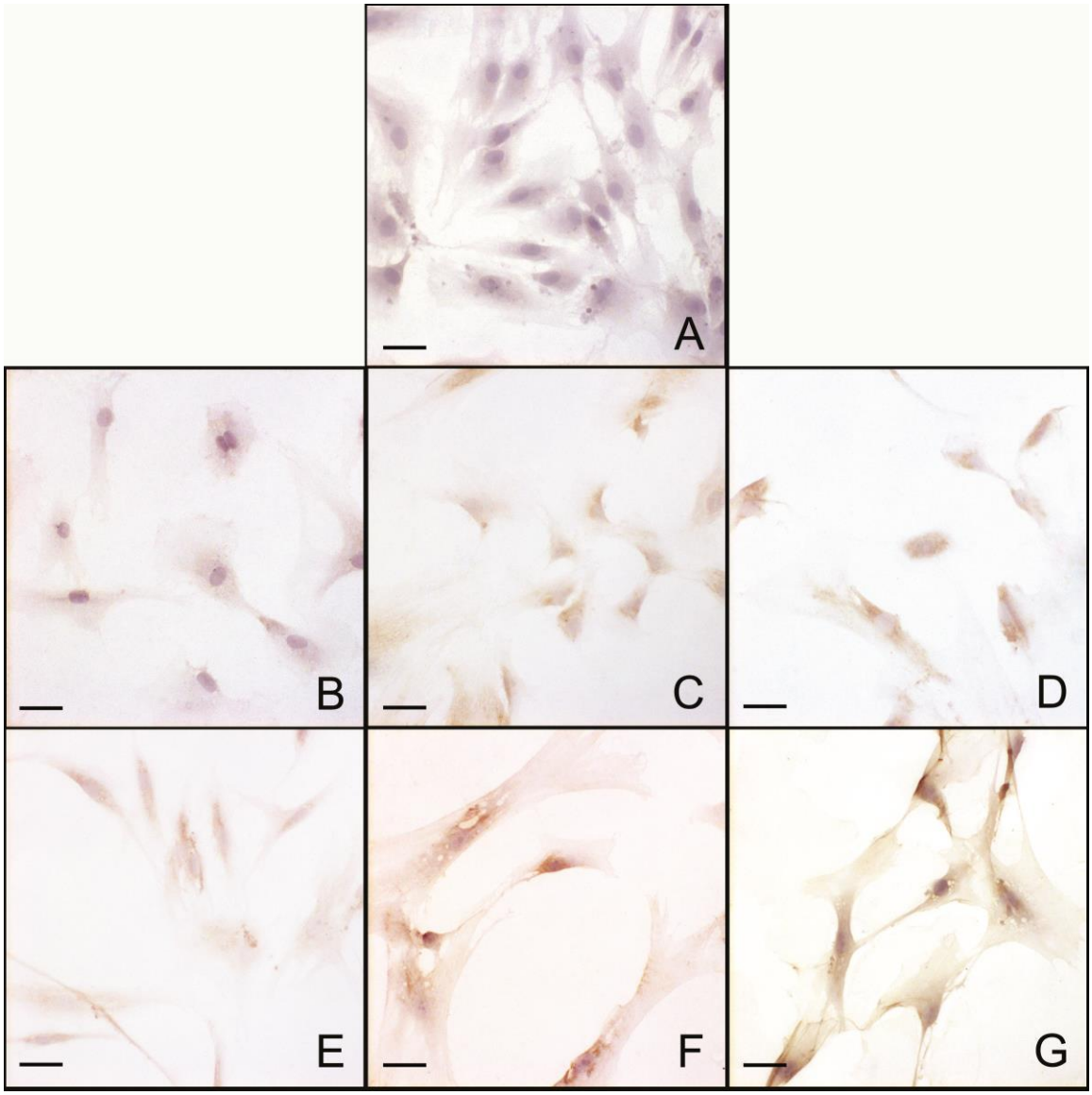

Figure 24: (A-G) Immunohistochemistry analysis (20X) for Osteopontin on HOBs comparing different experimental groups: A) Negative Control Group; B) Complete medium (OGM); C) OGM + Na_4SiO_4 0,5mM; D) OGM + Na_4SiO_4 1mM; E) OGM + CGF; F) OGM + CGF + Na_4SiO_4 0,5 mM; G) OGM + CGF + Na_4SiO_4 1mM (Bar=40µm).

5. DISCUSSION

The present *in vitro* study showed that Silicon, in the soluble form of Sodium Orthosilicate (Na_4SiO_4) and the platelet concentrate CGF, had a positive effect on the growth, proliferation and metabolic activity, in all the three human cell lines used (NHDF, HUVEC and HOBs), even if better results were obtained for endothelial cells. In NHDF, FACS analysis for the quantification of the intracellular proliferation marker ki-67, showed that treatment with Sodium Orthosilicate and CGF did not significantly influence cell growth and proliferation, even if the percentage of Ki-67 positive cells was higher in cells treated with both CGF and Na_4SiO_4, respect to complete medium (FGM) supplemented and not with Na_4SiO_4. Also the immunohistochemistry for Collagen type 1 showed that immunopositivity progressively increased in NHDF treated with CGF and Na_4SiO_4, being statistically significant compared with FGM supplemented and not with Na_4SiO_4. In literature there are several studies which suggest a beneficial effect of Silicon in skin and its appendages (*Wickett et al., 2007; Barel et al., 2005; Refitt et al., 2003*). In particular, it is reported that Silicon is important for optimal synthesis of collagen and for activating the hydroxylation enzymes, improving skin strength and elasticity. According to Refitt and colleagues (*Refitt et al., 2003*), Collagen type 1 was significantly increased in cultures of skin fibroblasts treated with orthosilicic acid at 10 and 20 μm. This is probably due to the Silicon biological mechanism of action. In fact, an increase in Silicon concentration in the blood is progressively followed by an increase in the maturation of L-proline to Hydroxyproline, leading to a more efficient synthesis of collagen. All this suggests that, acting on the synthesis of collagen and glycosaminoglycans, we can improve the mechanical properties and appearance of various tissues and organs, including the skin. As regards platelet concentrates, several studies (*Kushida et al., 2013;*

Kakudo et al., 2008; Liu et al., 2002) showed that the use of PRP on human dermal fibroblasts markedly increased cell proliferation, supporting the clinical application of platelet preparations for cell-based wound repair and regeneration. These data were in agreement with the results obtained in the present work, in which treatment with Sodium Orthosilicate and CGF, markedly increase Collagen type I levels in human fibroblasts. A possible explanation of this effect could be that platelet preparations are rich in growth factors that once released, play an important role in NHDF growth and proliferation. In fact fibroblast proliferation can be induced by tumour necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β) and also by others factors that are present in platelets preparations. Moreover, TGF-β can induce collagen type 1 synthesis (*Ohji et al., 1993)* increasing the secretion of the enzyme collagenase. So the combination of CGF and Sodium Orthosilicate have a synergistic effect on NHDF growth and proliferation and markedly increased Collagen type 1 sinthesys.

Similar results were obtained for human edndothelial cells (HUVEC). Unlike NHDF, facs analysis for the quantification of the intracellular proliferation marker ki-67, showed that treatment with Sodium Orthosilicate and CGF significantly influenced HUVEC growth and proliferation. The highest expression of Ki-67 was observed in cells treated with CGF and Na_4SiO_4 0,5mM, even if a significantly amount was obtained also with CGF supplemented with Na_4SiO_4 1mM. Some evidences suggest a positive correlation between Silicon consumption and vascular homeostasis (*Schwarz et al., 1977*). In particular, it seems that dietary Si intake improves the cardiovascular system, being essential to the structural integrity, elasticity and permeability of the arteries and exerting a protective and preventive anti-atherosclerotic effect. In fact, immunohistochemistry for the angiogenetic factor VEGF and the vasoactive molecule eNOS, showed that immunopositivity

progressively increasd after Na_4SiO_4 treatment, reaching the highest amount in cells treated with CGF and Na_4SiO_4. As regards Silicon effect on endothelial cells, the exact mechanism of action is not fully known and requires further in-depth investigation. However, according to Buffoli and colleagues (*Buffoli et al., 2013*), Silicon consumption results in an increase of some vasoactive molecules involved in the processes of oxidative stress, the endothelial nitric oxide synthase (eNOS) and aquaporin-1 (AQP1) and could therefore act as a protective factor against vascular alterations. These data were in agreement with the results obtained in the present study. Moreover, it was reported that also platelet preparations such as PRP, promote angiogenesis both *in vivo* and *in vitro*, even if few studies have been published on the effects of platelet concentrates on endothelial cells. In the work of Kakudo and collaborators (*Kakudo et al., 2014*), the *in vitro* use of PRP, showed to induce the proliferation, migration and tube formation of vascular endothelial cells, that are major processes in angiogenes. Other evidences (*Bertrand-Duchesne et al., 2010; Frechette et al., 2005)* also demonstrated the mitogenic potential of PRP on HUVECs. The reason of this effect is probably because platelet concentrates are rich in different growth factors among which there are also powerful angiogenic factors such as VEGF and PDGF, which act synergistically, promoting angiogenesis. Moreover PRP is thought to participate in activation of the PI3K/AKT pathway. This pathway is known to play a key role in numerous cellular functions including proliferation, adhesion, migration, invasion, metabolism, survival and angiogenesis (*Bader et al., 2005*). The PI3K/AKT pathway increases VEGF secretion and it also regulates angiogenesis by modulating expression of nitric oxide (NO) and angiopoietins (ANG1 and ANG2). It has been reported that VEGF up-regulates the expression of eNOS in endothelial cells (*Bouloumié et al., 1999*), playing a key role in VEGF-induced angiogenesis and vascular permeability. Our data are in agreement

with these evidences, showing a significant increase in both VEGF and eNOS levels, after treatment with CGF and Na_4SiO_4.

Finally, as regards human osteoblast cells, facs analysis for the quantification of ki-67 percentage, showed that there were no statistical difference after cell treatment with Sodium Orthosilicate and CGF. This was probably due because these cells proliferate more quickly than NHDF and HUVEC and so when submitted to facs analysis the cells were not in active proliferation. In fact observing the cells at the end of the experimental period (72 hours), they were almost to confluence. On the contrary, immunohistochemistry for Collagen type 1 and Osteopontin showed that immunopositivity progressively increased among the different treatments, reaching the highest amount in HOBs treated with CGF and Na_4SiO_4. These results were in agreement with results obtained in the present study and with data present in literature. In fact, there are several studies performed both *in vitro* and *in vivo* (*Kim et al.,2013; Shie et al., 2011*), which support the beneficial effects of Silicon on bone cell growth and proliferation, increasing bone matrix synthesis and deposition and the osteoblasts metabolic activity. Kim and colleagues (*Kim et al., 2013*) analyzed the role of Silicon, in form of Sodium Metasilicate, on the MC3T3 murine cell line, showing an increase in bone formation and mineralization. An additional study (*Zou et al., 2009*) evaluated the effects of silicate ions treatment on human osteoblast cells (HOBs), showing an increase in cellular metabolic activity and proliferation. Studies in rats have demonstrated that Silicon at physiological levels improved calcium incorporation in bone when compared to rats that are Silicon deficient (*Seaborn et al., 2002; Rico et al., 2000; Hott et al., 1993*). Keting and colleagues (*Keeting et al., 1992*) showed that Zeolite A, a particulate material containing Silicon, stimulates the proliferation and differentiation of osteoblast-like cells in culture. Carlisle *(Carlisle, 1980)* found that silicon deprivation reduced the collagen content in skull and long

bone. The author also reported that silicon stimulated the activity of prolyl hydroxylase in frontal bones of chick embryos *in vitro (Carlisle 1976, 1972).* Refitt and collaborators (*Refitt et al., 2003)* demonstrated that physiological concentrations (10–20 μM) of soluble silicon stimulate collagen type I synthesis in human osteoblast-like cells and promote osteoblast differentiation. These evidences were in agreement with the results obtained. In fact immunopositivity for Collagen type 1 progressively increased after Sodium Orthosilicate treatment respect to complete medium alone. Similar trend was observed for Osteopontin, a prominent bone matrix protein that is synthesized by osteoblastic cells but also by several cell types other than bone cells, including hypertrophic chondrocytes, kidney proximal tubule epithelial cells, and arterial smooth muscle cells. In bone, Osteopontin is involved in bone cell attachment to the bone matrix and generates intracellular signals that affect osteoclast motility. An *in vitro* study in rats *(Nielsen et al., 2004),* showed that circulating osteopontin was decreased by both silicon deprivation and ovariectomy. Also *in vivo* and *in vitro* studies using artificial scaffolds containing Silicon, showed osteoconductive, osteoproductive and osteoinductive properties, increasing osteoblasts proliferation and differentiation. According to the results obtained in the present work, levels of Collagen type 1 and Osteopontin were significantly higher in HOBs treated with CGF and Sodium Orthosilicate, suggesting that the stimulatory effect of Silicon is probably potentiated by the addition of CGF. In fact there are several studies *(He et al., 2009;Kanno et al., 2005; Ogino et al., 2005)* that show a beneficial effect of platelet preparations (PRP, PRF and CGF) on osteoblasts growth, proliferation and differentiation, being these platelet preparations reach in growth factors. Among these growth factors, BMPs, FGFs, VEGFs, PDGFs and IGFs have significant impacts on osteoblast behavior, enhancing osteoblasts proliferation *(Yun et al., 2012)* and the expression of bone markers such as Collagen type 1 and Osteopontin

(*Ramchandani et al., 2015; Li et al., 2012*), and thus have been widely utilized for bone tissue regeneration

According to Ogino and colleagues (*Ogino et al., 2005*), PDGF and TFG-β released from PRP, contribute to the proliferation of osteoblastic-like cells. Ling and colleagues (*He et al., 2009*) compared the effect of PRF and PRP on the proliferation and differentiation of rats osteoblasts *in vitro*, showing that PRF released growth factors gradually and expressed stronger and more durable effect on proliferation and differentiation of rat osteoblasts that PRP. There are also several evidences (*Qiao et al. 2016; Wang et al., 2016; Takeda et al., 2015)* which suggest a beneficial role of CGF in bone regeneration. It is used also in combination with bioactive materials, such as beta Tricalcium Phosphate (β-TCP) and Bio-Oss (*Wang et al., 2016*) improving osteogenesis and so promoting new bone formation.

Overall the findings of the present study suggest that *in vitro* treatment with CGF and Sodium Orthosilicate seems to be promised in promoting cell growth and proliferation and so in tissue regeneration. On the basis of these results, animal studies should be performed to evaluate the regenerative capacity of CGF and Sodium Orthosilicate also in vivo.

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