RESEARCH ARTICLE

In vitro treatment with concentrated growth factors (CGF) and sodium orthosilicate positively affects cell renewal in three different human cell lines

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Abstract

The aim of this study was to investigate the in vitro effect of Silicon, in the soluble form of sodium orthosilicate, combined and not with the concentrated growth factors (CGF), a platelet-rich preparation, 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 sodium orthosilicate combined with CGF, for 72 h. At the end of the experimental period, the in vitro effect 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 Fluorescence-activated cell sorting (FACS) analysis. Moreover, the expression of other cell markers and active molecules, such as Collagen type I, Osteopontin, Vascular Endothelial Growth Factor, and endothelial Nitric Oxide Synthase, was evaluated, through immunohistochemistry. Results obtained showed that the combined use of CGF and sodium orthosilicate stimulates cell growth, proliferation, and metabolic activity, suggesting that this treatment could be effective in tissue regeneration.

Keywords: CGF; flow cytometry; growth factors; platelets; sodium orthosilicate; tissue engineering

Introduction

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. It is a complex process of healing and tissue growth, which involves different biological elements and strategies, such as the use of bone grafts (García-Gareta et al., 2015), biomaterials, growth factors, natural or synthetic scaffolds (Loh and Choong, 2013; Asti and Gioglio, 2014) and the use of autologous cells, in particular stem cells (Manunta et al., 2016; Joo et al., 2017). Recently, the use of platelet-rich 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 (Ramos-Torrecillas et al., 2014; Sermer et al., 2015; Badran et al., 2017). These concentrates are obtained from the patient’s own blood and contain autologous platelets, growth factors, and cytokines involved in the key processes

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Abbreviations: BC, buffy coat; BMPs, bone morphogenetic proteins; CGF, concentrated growth factors; Col I, collagen type I; EGF, epidermal growth factor; EGM, endothelial growth medium; eNOS, endothelial nitric oxide synthase; FGF, fibroblast growth factor; FGM, fibroblast growth medium; HOBs, human osteoblasts; HUVEC, human umbilical vein endothelial cells; IGF, insulin-like growth factor; Na4SiO4, sodium orthosilicate; NHDF, normal human dermal fibroblasts; OGM, osteoblast growth medium; OPN, osteopontin; OSA, orthosilicic acid; PDGF, platelet-derived growth factor; PPP, platelet poor plasma; PRGF, platelet-rich in growth factors; PRF, platelet-rich fibrin; PRP, platelet-rich plasma; RBC, red blood cells; RP, red part; Si, silicon; SiO2, silica; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor; WP, white part
of tissue regeneration, including cell proliferation and differentiation, extracellular matrix synthesis, chemotaxis, and angiogenesis (Borsani et al., 2015; Fioravanti et al., 2016). Platelets represent an important source of growth factors that are stored in their α-granules (Blair and Flaumenhaft, 2009). After activation, platelets release a multitude of growth factors at concentrations significantly higher than the baseline blood levels, including transforming growth factor beta (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), bone morphogenetic proteins (BMPs), and many others. Several evidences (Olalfe and Fadason, 2017; Paul et al., 2017; Yang et al., 2017; Zhang et al., 2017) suggest that also some trace elements play an important role in tissue growth and development and especially in bone metabolism and turnover. Among these, Silicon (Si) is an important trace element and increasing evidences (Bae et al., 2008; Kim et al., 2009; Zou et al., 2009; Refitt et al., 2003; Dong et al., 2017) suggest that Si dietary intake is positively correlated with bone homeostasis and regeneration, representing a potential and valid support for the prevention and improvement of bone diseases, like osteoporosis. This trace element represents the second most abundant element in the earth’s crust (28 %), after oxygen (47%), but it is rarely found in its elemental form due to its great affinity for oxygen, forming silica (SiO2) and silicates, which at 92%, are the most common minerals. Human are exposed to numerous sources of Si 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). The biological importance of Si is closely related to its bio-distribution. Tissue levels, however, vary. In rat, highest levels are found in bone and other connective tissues such as, skin, nail, hair, trachea, tendons, and aorta, whereas lower levels are found in soft tissues (Exley, 1998). A similar tissue Si distribution is expected in humans, although this has not been investigated in the human organism. As regards the mechanism, Si would seem to act on the enzyme prolyl hydroxylase (Carlisle et al., 1981; Carlisle and Alpenfels, 1984) 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. This biological catalyst plays a key role in the development of joints, bones, skin and skin appendages and therefore Si intake stimulates the synthesis of collagen (Refitt et al., 2003; Dong et al., 2017), leading to a harmonious development of connective and other tissues. Apart from its physiologically role in bone and cartilage formation, Si seems to be an essential element also for the brain, in which provides protection against aluminum (Al) accumulation and consecutive oxidative damage (Foglio et al., 2012; Davenward et al., 2013), for cardiovascular system promoting angiogenesis (Fielding and Bose, 2013) for skin, hair, and nails, improving skin surface and mechanical properties and brittleness of hair and nails (Fregert, 1958; Bonazza et al., 2016).

Based on these evidences, the aim of the present study was to investigate the in vitro effect of silicon, in the soluble form of sodium orthosilicate, combined and not with the platelet concentrate CGF, on the proliferation and metabolic activity of three different human cell lines: normal human dermal fibroblasts (NHDF), human umbilical vein endothelial cells (HUVEC), and human osteoblasts (HOBs).

Materials and methods

Human cell lines

For in vitro experiments with sodium orthosilicate (Na4SiO4), CGF, and Na4SiO4 combined with CGF, three different cryopreserved human cell lines were used: 1) normal human dermal fibroblasts (NHDF, cell derived from skin of adult donor; Lonza, USA) that are the main cells of connective tissue responsible for the collagen and mucopolysaccharides production, for the deposition of extra cellular matrix and the maintenance, degradation and rearrangement of its structure, through the production of protein molecules including laminin and fibronectin; 2) human umbilical vein endothelial cells (HUVEC, pooled cells, Lonza, USA) that 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; 3) human osteoblast cells (HOBs, cryopreserved cells, Promocell, Germany) that are highly specialized cell type, of mesenchymal origin, involved in bone formation and remodeling.

Sodium orthosilicate preparation

Silicon in the form of Na4SiO4 (Alfa Aesar, Germany) was used, because readily soluble in water, producing an alkaline solution. A stock solution of Na4SiO4 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 a heating plate and a magnet. The pH solution was checked using a calibrated pH meter and it was 15. Nitric Acid 10 M (VWR International, Milan) was added to lower the pH solution to 7. Once prepared, the stock solution was filtered using a 0.2 μm syringe filter, under a laminar flow cabinet and two different concentrations of Na4SiO4 (0.5 and
1 mM) were prepared in cell culture media by serial dilution of the 0.1 M stock solution, according to Refitt et al. (2003).

CGF preparation

CGF was obtained by collecting whole blood from three healthy adult volunteers, consisting of one man and two women, aged 28–53 years. Exclusion criteria were a systemic disorder, smoking, infection, non-steroidal anti-inflammatory drug use, and a hemoglobin level of <11 g/dL or a platelet concentration of <150,000 mm$^3$. Informed consent was obtained from all individual participants included in the study. Blood was collected using specific tubes coated with silica micro-particles (sterile Vacuette tubes, Greiner Bio-One, GmbH, Kremsmunster, Austria) and a special programmed centrifuge (Medifuge MF200, Silfradent srl, Forli, Italy). At the end of the centrifugation process, three blood fractions were identified (Borsani et al., 2015; Bonazza et al., 2016): the upper platelet poor plasma layer (PPP), representing the liquid phase of plasma; the lower red blood cells layer (RBC), containing erythrocytes and the middle CGF layer, consisting into three parts: the upper white part (WP), the downer red part (RP), and the middle “buffy coat” part (BC), representing the interface between WP and RP and containing leukocytes and platelets. Once obtained, CGFs were removed from each tube with sterile tweezers, under a laminar flow cabinet and the lower part of RBC was cut using sterile scissors. Subsequently, each whole CGF was processed in relation to the experiment. The research was conducted according to the principles of the Declaration of Helsinki.

Cell culture and treatments

Cells (NHDF, HUVEC, and HOBs) were grown in their specific complete medium (FGM—fibroblast growth medium, for NHDF; EGM—endothelial growth medium for HUVEC, and OGM—osteoblast growth medium for HOBS) until they reached 80% of confluence, changing the medium every 2 days. Then cells were seeded, at a final density of 10,000 cells/cm$^2$, in 6-well culture plates (Sarstedt, Nuembrecht, Germany) and starved in basal medium for 24 h. After the starvation period, basal medium was replaced with the specific complete medium and the cells were subjected to different treatments for 72 h.

In particular, NHDF were subjected to the following treatments: 1) Fibroblast complete medium alone (FGM), which represents the control; 2) FGM supplemented with sodium orthosilicate 0.5 mM (FGM + Na$_4$SiO$_4$ 0.5 mM); 3) FGM supplemented with sodium orthosilicate 1 mM (FGM + Na$_4$SiO$_4$ 1 mM); 4) FGM combined with CGF (FGM + CGF); 5) FGM combined with CGF and sodium orthosilicate 0.5 mM (FGM + CGF + Na$_4$SiO$_4$ 0.5 mM); 6) FGM combined with CGF and sodium orthosilicate 1 mM (FGM + CGF + Na$_4$SiO$_4$ 1 mM).

HUVEC were subjected to the same treatments using their specific complete medium EGM: 1) Endothelial complete medium alone (EGM), which represents the control; 2) EGM supplemented with sodium orthosilicate 0.5 mM (EGM + Na$_4$SiO$_4$ 0.5 mM); 3) EGM supplemented with sodium orthosilicate 1 mM (EGM + Na$_4$SiO$_4$ 1 mM); 4) EGM combined with CGF (EGM + CGF); 5) EGM combined with CGF and sodium orthosilicate 0.5 mM (EGM + CGF + Na$_4$SiO$_4$ 0.5 mM); 6) EGM combined with CGF and sodium orthosilicate 1 mM (EGM + CGF + Na$_4$SiO$_4$ 1 mM).

HOBs were subjected to the same treatments using their specific complete medium OGM: 1) Osteoblast complete medium alone (OGM), which represents the control; 2) OGM supplemented with sodium orthosilicate 0.5 mM (OGM + Na$_4$SiO$_4$ 0.5 mM); 3) OGM supplemented with sodium orthosilicate 1 mM (OGM + Na$_4$SiO$_4$ 1 mM); 4) OGM supplemented with CGF (OGM + CGF); 5) OGM combined with CGF and sodium orthosilicate 0.5 mM (OGM + CGF + Na$_4$SiO$_4$ 0.5 mM); 6) OGM combined with CGF and sodium orthosilicate 1 mM (OGM + CGF + Na$_4$SiO$_4$ 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 h. At the end of the treatment period, the in vitro effect on cell growth, proliferation and activity was evaluated through FACS analysis, cell count and immunohistochemistry.

FACS analysis

FACS analyses were performed on cells following a specific protocol: first of all, cells were detached using the t trypsin (0.025%)/EDTA (0.01%) solution (Promocell, Heidelberg, Germany) and centrifuged at 1,000 rpm for 5 min (Borsani et al., 2015). Subsequently, the supernatant was discarded, whereas pellet was re-suspended in the specific culture medium. 100–200 μL of cell suspension were transferred into each fresh tube and permeabilized with saponin (1 mL/ tube), on ice for 10 min. Then cells were centrifuged at 1,200 rpm for 5 min and the supernatant was removed. Twenty microliter of Ki-67 mouse monoclonal antibody Ki-67 FITC-conjugated (BD Bioscience, San Diego, CA) were added to each tube and incubated in the dark for 30 min, at 4°C. The Ki-67 isotype control (BD Bioscience, San Diego, CA) was used as positive control and as negative control the
primary antibody was omitted and only a secondary FITC antibody was used. After washing the cells with FACS buffer (PBS with 2% FBS), having centrifuged and re-suspended them in 200–300 μL of FACS buffer, 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).

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 1,000 rpm for 5 min. After removing the supernatant, pellet was re-suspended in the appropriate culture medium. Cell suspension (200 μL) was transferred into an Eppendorf tube of 2 mL, with a round base for Scepter cell counter. Depending on the cell diameter, a specific sensor (40 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.

Immunohistochemistry

At the end of the culture period, the different cell types were fixed on coverslips to perform immunohistochemistry of some cell markers and in particular collagen type I (Col I), osteopontin (OPN), vascular endothelial growth factor (VEGF), and endothelial nitric oxide synthase (eNOS). Immunohistochemistry was performed using the UltraVision Quanto Detection System Horseradish Peroxidase (HRP; ThermoScientific, Bio-Optica, Milan, Italy), followed by development with the chromogen substrate 3,3′-diaminobenzidine (DAB, Amresco, Prodotti Gianni, Milan, Italy). Before adding the mouse monoclonal antibody, the cells were permeabilized with Triton 0.1% for 10 min and then incubated with blocking solution for 5 min. Then the different primary antibodies were added. To better visualize the positive reaction, the cells were counterstained with Carazzi’s Hematoxylin, dehydrated, and mounted with DPX, for light microscopy detection. 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 normalized to cell number.

Statistical analysis

All experiments were done in three independent experiments and 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. A value of \( P < 0.05 \) was considered statistically Significant. Origin v9.0 software was used for all statistical analyses performed.

Figure 1  FACS analysis after 72 h of treatment to quantify the expression of the intracellular proliferation marker Ki-67 in (A) NHDF: graph comparing six experimental groups: FGM (complete medium), which represents the control; FGM + Na4SiO4 0.5 mM; FGM + Na4SiO4 1 mM; FGM + CGF; FGM + CGF + Na4SiO4 0.5 mM; FGM + CGF + Na4SiO4 1 mM. The Ki-67 positive cells are reported as % ± SE. (B) HUVEC: graph comparing six experimental groups: EGM (complete medium), which represents the control; EGM + Na4SiO4 0.5 mM; EGM + Na4SiO4 1 mM; EGM + CGF; EGM + CGF + Na4SiO4 0.5 mM; EGM + CGF + Na4SiO4 1 mM. The Ki-67 positive cells are reported as % ± SE. (C) HOBs: graph comparing six experimental groups: OGM (complete medium), which represents the control; OGM + Na4SiO4 0.5 mM; OGM + Na4SiO4 1 mM; OGM + CGF; OGM + CGF + Na4SiO4 0.5 mM; OGM + CGF + Na4SiO4 1 mM. The Ki-67 positive cells are reported as % ± SE.
Table 1 Cell count on NHDF after the different treatments. Data are shown as the mean ± SE.

<table>
<thead>
<tr>
<th></th>
<th>24 h pre-treatment</th>
<th>72 h treatment</th>
<th>Cell number/mL</th>
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<tbody>
<tr>
<td>FBM</td>
<td>FGM</td>
<td>2.46 × 10^6 ± 0.2</td>
<td></td>
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<tr>
<td>FBM</td>
<td>FGM + Na₄SiO₄ 0.5 mM</td>
<td>3.80 × 10^6 ± 0.3</td>
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<tr>
<td>FBM</td>
<td>FGM + Na₄SiO₄ 1 mM</td>
<td>3.90 × 10^6 ± 0.3</td>
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<tr>
<td>FBM</td>
<td>CGF</td>
<td>4.27 × 10^6 ± 0.3</td>
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<tr>
<td>FBM</td>
<td>CGF + Na₄SiO₄ 0.5 mM</td>
<td>5.22 × 10^6 ± 0.4</td>
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<tr>
<td>FBM</td>
<td>CGF + Na₄SiO₄ 1 mM</td>
<td>6.23 × 10^6 ± 0.4</td>
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**Results**

Percentage of Ki-67 positive cells was higher in cells treated with Na₄SiO₄ and CGF

To determine the in vitro effect of treatment with Na₄SiO₄ and CGF on cell growth and proliferation, we performed FACS analyses on NHDF, HUVEC, HOBs for the quantification of the intracellular proliferation marker ki-67 and we also performed a simple cell count, using an automated cell counter (Scepter™ 2.0 Cell Counter, Millipore) in order to establish the total number of cells.

FACS analysis in NHDF cells showed that treatment for 72 h, with Na₄SiO₄ and CGF not significantly influenced cell growth and proliferation (Figure 1A). Similar results were obtained performing a simple cell count (Table 1), using an automated cell counter (Scepter™ 2.0 Cell Counter, Millipore): FGM alone (2.46 × 10^6 ± 0.2), FGM + Na₄SiO₄ 0.5 mM (3.80 × 10^6 ± 0.3), FGM + Na₄SiO₄ 1 mM (3.90 × 10^6 ± 0.3), FGM + CGF (4.27 × 10^6 ± 0.3), FGM + CGF + Na₄SiO₄ 0.5 mM (5.22 × 10^6 ± 0.4), and FGM + CGF + Na₄SiO₄ 1 mM (6.23 × 10^6 ± 0.4).

FACS analysis in HUVEC cells showed that treatment with CGF and sodium orthosilicate significantly influenced cell growth and proliferation (Figure 1B). In fact, the highest percentage of Ki-67 positive cells was observed in cells treated with EGM + CGF + Na₄SiO₄ 0.5 mM (90.2% ± 2.7) and EGM + CGF + Na₄SiO₄ 1 mM (86.4% ± 2). These treatments were statistically different compared with EGM (67.3% ± 1.2), EGM + Na₄SiO₄ 0.5 mM (77.6% ± 0.5) and EGM + Na₄SiO₄ 1 mM (70.7% ± 0.6). Similar results were obtained performing a simple cell count (Table 2) using an automated Scepter™ 2.0 Cell Counter (Millipore). The number of cells (Table 2) progressively increased in EGM + CGF (4.20 × 10^6 ± 0.2), EGM + CGF + Na₄SiO₄ 0.5 mM (4.95 × 10^6 ± 0.6) and EGM + CGF + Na₄SiO₄ 1 mM (4.60 × 10^6 ± 0.5), compared with EGM alone (2.9 × 10^5 ± 0.2), EGM + Na₄SiO₄ 0.5 mM (3.80 × 10^5 ± 0.5) and EGM + Na₄SiO₄ 1 mM (3.1 × 10^5 ± 0.5).

FACS analysis in HOBs showed similar results to those obtained for fibroblasts and so treatment with CGF and sodium orthosilicate not significantly influenced cell growth and proliferation (Figure 1C). Similar results were obtained performing a simple cell count (Table 3) with the automated Scepter™ 2.0 Cell Counter (Millipore): OGM alone (9.64 × 10^4 ± 0.4), OGM + Na₄SiO₄ 0.5 mM (1.03 × 10^5 ± 0.3), OGM + Na₄SiO₄ 1 mM (1.31 × 10^5 ± 0.2), OGM + CGF (1.70 × 10^5 ± 0.2), OGM + CGF + Na₄SiO₄ 0.5 mM (1.72 × 10^5 ± 0.1), OGM + CGF + Na₄SiO₄ 1 mM (1.75 × 10^5 ± 0.1).

A significant positive immunostaining for Col I was observed in NHDF and HOBs, after treatment with Na₄SiO₄ and CGF

Immunohistochemistry for Col I was performed on NHDF and HOBs. Positive immunostaining for Col I was observed in NHDF and HOBs, after treatment with Na₄SiO₄ and CGF (Figures 2A and 2B). In fact, in complete medium alone (FGM), immunopositivity was lower (20.8 ± 0.2), compared with the other experimental conditions. In FGM + Na₄SiO₄ 0.5 mM immunopositivity (21.6 ± 0.4) was similar to FGM but lower than FGM + Na₄SiO₄ 1 mM (23.3 ± 0.4). Immunopositivity for Col I, progressively increased in FGM + CGF (26.1 ± 1.6), FGM + CGF + Na₄SiO₄ 0.5 mM (27.7 ± 0.6) and FGM + CGF + Na₄SiO₄ 1 mM (29.1 ± 1.2), being statistically significant compared with FGM, FGM + Na₄SiO₄ 0.5 mM and FGM + Na₄SiO₄ 1 mM.

Similar results were obtained for HOBs. The intensity of the reaction increased progressively among the different

Table 2 Cell count on HUVEC after the different treatments. Data are shown as the mean ± SE.

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<td>EBM</td>
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<tr>
<td>EBM</td>
<td>EGM + Na₄SiO₄ 1 mM</td>
<td>3.1 × 10^5 ± 0.5</td>
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<td>EBM</td>
<td>CGF</td>
<td>4.20 × 10^5 ± 0.2</td>
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<tr>
<td>EBM</td>
<td>CGF + Na₄SiO₄ 0.5 mM</td>
<td>4.95 × 10^5 ± 0.6</td>
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<tr>
<td>EBM</td>
<td>CGF + Na₄SiO₄ 1 mM</td>
<td>4.60 × 10^5 ± 0.5</td>
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</table>

Table 3 Cell count on HOBs after the different treatments. Data are shown as the mean ± SE.

<table>
<thead>
<tr>
<th></th>
<th>24 h pre-treatment</th>
<th>72 h treatment</th>
<th>Cell number/mL</th>
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<td>OBM</td>
<td>OGM</td>
<td>9.64 × 10^4 ± 0.4</td>
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<tr>
<td>OBM</td>
<td>OGM + Na₄SiO₄ 0.5 mM</td>
<td>1.03 × 10^5 ± 0.3</td>
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<td>OBM</td>
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<tr>
<td>OBM</td>
<td>CGF + Na₄SiO₄ 1 mM</td>
<td>1.75 × 10^5 ± 0.1</td>
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treatments (Figures 3A and 3B). In fact, in complete medium alone (OGM), immunopositivity for Col I was lower 
(10.01 ± 0.4), compared with the other experimental conditions. In OGM + Na₄SiO₄ 0.5 mM, immunopositivity was slightly higher (14.96 ± 0.6) compared with OGM but lower respect to the other treatments. In OGM + Na₄SiO₄ 1 mM, there was a small increase in Col I immunopositivity (16.65 ± 0.3) respect to OGM + Na₄SiO₄ 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 + Na₄SiO₄ 0.5 mM and OGM + Na₄SiO₄ 1 mM but it was lower respect to OGM + CGF + Na₄SiO₄ 0.5 mM (24.54 ± 0.7) and OGM + CGF + Na₄SiO₄ 1 mM (26.16 ± 0.5). Both in OGM + CGF + Na₄SiO₄ 0.5 mM and OGM + CGF + Na₄SiO₄ 1 mM, immunopositivity greatly increased, being statistically significant respect to all the other treatments.

A significant positive immunostaining for OPN was observed in HOBs after treatment with sodium orthosilicate (Na₄SiO₄) and CGF

Immunohistochemical analysis for OPN was performed on HOBs. OPN immunopositivity was observed in osteoblast cells and it showed a similar trend to Col I, progressively increasing among the different treatments (Figures 3C and 3D). In OGM + Na₄SiO₄ 0.5 mM (19.45 ± 0.5) and OGM + Na₄SiO₄ 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 Na₄SiO₄. In OGM + CGF immunopositivity markedly increased (23.41 ± 0.9), being statistically different from OGM. OGM + CGF + Na₄SiO₄ 0.5 mM (25.97 ± 0.6) and OGM + CGF + Na₄SiO₄ 1 mM (26.05 ± 0.6), showed a very similar amount in OPN (even if it was slightly higher in CGF + Na₄SiO₄ 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.

A significant positive immunostaining for VEGF and eNOS was observed in HUVEC after treatment with sodium orthosilicate (Na₄SiO₄) and CGF

Immunohistochemical analysis for VEGF and eNOS was performed on HUVEC. A positive immunostaining for VEGF was observed in endothelial cells (Figures 4A and 4B)
and immunopositivity significantly increased in cells treated with CGF and Na$_4$SiO$_4$ 0.5 mM and 1 mM. In EGM + Na$_4$SiO$_4$ 0.5 mM (35.15 ± 0.4) and EGM + Na$_4$SiO$_4$ 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$_4$SiO$_4$ 0.5 mM and EGM + Na$_4$SiO$_4$ 1 mM, but lower than EGM + CGF + Na$_4$SiO$_4$ 0.5 mM and EGM + CGF + Na$_4$SiO$_4$ 1 mM. EGM + CGF + Na$_4$SiO$_4$ 0.5 mM (46.84 ± 0.8) and EGM + CGF + Na$_4$SiO$_4$ 1 mM (47.5 ± 1.2) showed a similar immunopositivity which was significantly higher compared with the other treatments but there was no statistical difference between these two treatments.

Similar results were obtained for eNOS immunohistochemistry (Figures 4C and 4D). Immunopositivity significantly increased in cells treated with CGF and Na$_4$SiO$_4$ 0.5 mM and 1 mM. In EGM + Na$_4$SiO$_4$ 0.5 mM (34.75 ± 0.7) and EGM + Na$_4$SiO$_4$ 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$_4$SiO$_4$ 0.5 mM and EGM + Na$_4$SiO$_4$ 1 mM, but lower than EGM + CGF + Na$_4$SiO$_4$ 0.5 mM and EGM + CGF + Na$_4$SiO$_4$ 1 mM. EGM + CGF + Na$_4$SiO$_4$ 0.5 mM (47.43 ± 1.2) and EGM + CGF + Na$_4$SiO$_4$ 1 mM (48.64 ± 1.1) showed a similar immunopositivity which was significantly higher compared with the other treatments but there were no statistical differences between these two treatments.

**Discussion**

The present in vitro study showed that the combination of Si, in its soluble form of sodium orthosilicate (Na$_4$SiO$_4$) 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 Na$_4$SiO$_4$ and CGF did not significantly influence cell growth and proliferation. On the contrary, immunohistochemistry for Col I showed that immunopositivity progressively increased in NHDF treated with Na$_4$SiO$_4$ and CGF, being statistically significant compared with complete medium (FGM) supplemented and not with Na$_4$SiO$_4$. In literature, there are several studies which suggest a beneficial effect of Si in skin and its appendages (Fregert, 1958; Barel et al., 2005). In particular, it is reported that Si is important for optimal synthesis of collagen and for activating the hydroxylation enzymes, improving skin strength and elasticity. According to Refitt et al. (2003), Col I was significantly increased in cultures of skin fibroblasts treated with orthosilicic acid at 10 and 20 μM. This is probably due to the Si biological mechanism of action. In fact, Si would seem to act on the enzyme prolyl hydroxylase (Carlisle et al., 1981; Carlisle and Alpenfels, 1984) 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. This biological catalyst plays a key role in the development of joints, bones, skin and skin appendages and therefore Si intake stimulates the synthesis of collagen (Refitt et al., 2003; Dong et al., 2017; Figure 5). 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 (Kakudo et al., 2008; Kushida et al., 2013) showed that the use of PRP on human dermal fibroblasts markedly

**Figure 5** Possible effects of treatment with sodium orthosilicate and CGF on the three different human cell lines used: (a) Si and CGF on NHDF: Si treatment seems to act on the enzyme prolyl hydroxylase, allowing the conversion of l-proline amino acid into hydroxyproline leading to a more efficient synthesis of collagen (Refitt et al., 2003; Dong et al., 2017); CGF treatment provides growth factors such as TGF-β that induces Col I synthesis (Ohji et al., 1993) increasing the secretion of the enzyme collagenase; (b) Si and CGF on HUVEC: Si treatment seems to increase some vasoactive molecules involved in the processes of oxidative stress (eNOS and AQP1) and could therefore act as a protective factor against vascular alterations (Buffoli et al., 2013); CGF contains powerful angiogenic factors (VEGF and PDGF), which act synergistically, promoting angiogenesis; (c) Si and CGF on HOBs: Si stimulates the activity of prolyl hydroxylase (Carlisle et al., 1981; Carlisle and Alpenfels, 1984), stimulating Col I synthesis in human osteoblast-like cells; CGF contains osteogenic growth factors (BMPs, VEGFs, PDGFs, and IGFs) that enhances osteoblasts proliferation (Yun et al., 2012) and the expression of bone markers such as Col I and OPN (Ramchandani and Weber, 2015).
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 Na4SiO4 and CGF markedly increased Col I levels in human fibroblasts. A possible explanation of this effect could be that platelet preparations such as CGF, 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 tumor 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 Col I synthesis (Ohji et al., 1993) increasing the secretion of the enzyme collagenase. So, the combination of Na4SiO4 and CGF showed a synergistic effect on NHDF growth and proliferation and markedly increased Col I synthesis (Figure 5).

Similar results were obtained for human endothelial cells (HUVEC). Unlike NHDF, FACS analysis showed that treatment with Na4SiO4 and CGF significantly influenced HUVEC growth and proliferation. The highest expression of Ki-67 was observed in cells treated with Na4SiO4 0.5 mM and CGF, even if a significantly amount was obtained also with CGF supplemented with Na4SiO4 1 mM. Some evidences suggest a positive correlation between Si consumption and vascular homeostasis (Schwarz, 1977). In particular, it seems that dietary Si intake improves the cardiovascular system, being essential to the structural integrity, elasticity and permeability of the arterial and exerts a protective and preventive anti-atherosclerotic effect. In fact, immunohistochemistry for the angiogenic factor VEGF and the vasoactive molecule eNOS showed that immunopositivity progressively increased after Na4SiO4 treatment, reaching the highest amount in cells treated with Na4SiO4 and CGF. As regards Si effect on endothelial cells, the exact mechanism of action is not fully known and requires further in-depth investigation. However, according to Buffoli et al. (2013), Si 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 (Figure 5). 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 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 angiogenesis. Other evidences (Fréchette et al., 2005; Bertrand-Duchesne et al., 2010) also demonstrated the mitogenic potential of PRP on HUVEC. 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, which is known to play a key role in numerous cellular functions including proliferation, adhesion, migration, invasion, metabolism, survival, and angiogenesis. 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 Na4SiO4 and CGF (Figure 5).

Finally, as regards human osteoblast cells, FACS analysis showed that there was no statistical difference after cell treatment with Na4SiO4 and CGF. On the contrary, immunohistochemistry for Col I and OPN showed that immunopositivity progressively increased among the different treatments starting from complete medium alone (OGM) and reaching the highest amount in HOBs treated with Na4SiO4 and CGF. 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 (Rico et al., 2000; Kim et al., 2013), which support the beneficial effects of Si on bone cell growth and proliferation, increasing bone matrix synthesis and deposition and the osteoblasts metabolic activity. Kim et al. (2013) analyzed the role of Si, in form of sodium metasilicate, on the MC3T3 murine cell line, showing an increase in bone formation and mineralization. Studies in rats have demonstrated that Si at physiological levels improved calcium incorporation in bone when compared to Si deficient rats (Rico et al., 2000). 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 (1980a,b) found that Si deprivation reduced the collagen content in skull and long bone. The author also reported that Si stimulated the activity of prolyl hydroxylase in frontal bones of chick embryos in vitro (Carlisle, 1976). Refitt et al. (2003) demonstrated that physiological concentrations (10–20 µM) of soluble Si stimulate Col I synthesis in human osteoblast-like cells and promote osteoblast differentiation. These evidences were in agreement with the results obtained. In fact, immunopositivity for Col I progressively increased after Na4SiO4 treatment respect to complete medium alone. Similar trend was observed for OPN, 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, OPN 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 and Poellot, 2004) showed that circulating OPN was decreased by both Si deprivation and ovariectomy. Also in vivo and in vitro studies using artificial Si scaffolds showed osteoconductive, osteoprotective, and osteoinductive properties, increasing osteoblasts proliferation and differentiation. According to the results obtained in the present work, Col I and OPN levels were significantly higher in HOBs treated Na₄SiO₄ and CGF, suggesting that the Si stimulatory effect is probably potentiated by the addition of CGF (Figure 5). In fact, there are several studies (Kanno et al., 2005; He et al., 2009) 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 Col I and OPN (Rachandani and Weber, 2015) and thus have been widely utilized for bone tissue regeneration (Figure 5). There are also several evidences (Qiao and An, 2017; Wang et al., 2017a), 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., 2017b) improving osteogenesis and so promoting new bone formation.

**Conclusion**

Overall, the findings of the present study suggest that in vitro combined treatment with sodium orthosilicate and CGF seems to be efficient in promoting cell growth and proliferation and so in tissue regeneration. On the basis of these results, animal studies should be performed to confirm these data and to evaluate the regenerative capacity of sodium orthosilicate and CGF also in vivo.

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**Compliance with ethical standards**

The research was conducted according to the principles of the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

**Conflict of interest**

The authors declare that they have no conflicts of interest.

**References**


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