Osteosarcoma tissue-engineered model challenges oxidative stress therapy revealing promoted cancer stem cell properties

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A B S T R A C T

The use of oxidative stress generated by Cold Atmospheric Plasma (CAP) in oncology is being recently studied as a novel potential anti-cancer therapy. However, the beneficial effects of CAP for treating osteosarcoma have mostly been demonstrated in 2-dimensional cultures of cells, which do not mimic the complexity of the 3-dimensional (3D) bone microenvironment. In order to evaluate the effects of CAP in a relevant context of the human disease, we developed a 3D tissue-engineered model of osteosarcoma using a bone-like scaffold made of collagen type I and hydroxyapatite nanoparticles. Human osteosarcoma cells cultured within the scaffold showed a high capacity to infiltrate and proliferate and to exhibit osteomimicry in vitro. As expected, we observed significantly different functional behaviors between monolayer and 3D cultures when treated with Cold Plasma-Activated Ringer’s Solution (PAR). Our data reveal that the 3D environment not only protects cells from PAR-induced lethality by scavenging and diminishing the amount of reactive oxygen and nitrogen species generated by CAP, but also favours the stemness phenotype of osteosarcoma cells. This is the first study that demonstrates the negative effect of PAR on cancer stem-like cell subpopulations in a 3D biomimetic model of cancer. These findings will allow to suitably re-focus research on plasma-based therapies in future.

1. Introduction

Osteosarcoma (OS) is the most frequent primary bone cancer, characterized by high rates of tumor relapse and metastasis, that affects mostly children and young adults [1,2]. Although standard chemotherapy has significantly improved long-term survival, the outcome for those patients with recurrent or metastatic OS remains poor [2,3]. Thus, OS is still a therapeutic challenge and there is an urgent need to develop an effective therapy for these patients.

Cancer stem-like cells (CSLC) are a subpopulation of the tumor bulk with an indefinite capacity for division and self-renewal, which also retain a certain potential for differentiation to originate the different tumor subpopulations [4]. A number of studies demonstrate that CSLC play a pivotal role in tumor relapse since they are resistant to cytotoxic drugs through a number of mechanisms such as the adoption of a quiescent state, expression of ABC drug transporters, overexpression of anti-apoptotic proteins or the mediation of the response to DNA damage [5-7]. In the CSLC subpopulation also resides the ability of the tumor for metastasizing and colonizing new niches [7]. The capacity of CSLC to resist drugs and to regenerate the bulk of the tumor after chemotherapy gives rise to a different subpopulation of cancer cells within the tumor, exhibiting different drug sensitivities. This phenomenon is known as intratumor heterogeneity and it is the main challenge that therapies have to face to efficiently cure the disease [8].

It is known that the bone microenvironment plays a crucial role in
the maintenance of drug-resistant CSLC subpopulations and in the development and progression of OS [2,8–12]. Unfortunately, most cancer research has been done in 2D cultures, which lacks the 3D tissue microenvironment; as a result, less than 5% of the effective drugs tested in 2D models and preclinical mouse models succeed in clinical trials [12–15]. As an alternative to 2D models, bioengineered tumor models are being developed to more closely mimic the human 3D tumor microenvironment [16]. Bioengineered tumor models that are able to mimic the tumor environment are considered as invaluable tools to test new therapies against bone cancers [12,16–18].

In order to mimic in vitro the human 3D bone microenvironment, it is essential to include the cell compartment and the extracellular matrix (ECM). The human bone ECM is made of calcified bone matrix, which consists of about 60 wt% of inorganic phase mainly in the form of hydroxyapatite (HA), 30 wt% of organic phase (with type I collagen being the most abundant), and 10 wt% of water [19,20]. HA is of great therapeutic interest for bone repair modulation, due to its excellent biocompatibility, chemical similarity to native bone mineral and its capability to enhance cell attachment, proliferation, and differentiation of bone cells [19–21].

In the last years, the use of Cold Atmospheric Plasmas (CAP) to treat tumors is being investigated as a novel anti-cancer therapy [22,23]. CAP is an ionized gas at near-room temperature composed of a high number of reactive oxygen and nitrogen species (RONS), ions, electrons, metastable particles, electromagnetic field, and weak UV and VIS radiation [24]. CAP can be applied directly to cell cultures and superficial tissues, like the head and neck superficial tumors [22,23]. However, due to the plasma jet specifications, it is more complicated to apply CAP to the tumors located in deep tissues like bone without invasive surgery. To avoid this limitation saline solutions are treated with CAP and are under investigation to subsequently inject the CAP-treated solution locally in the tumor site. For instance, a recent study using a plasma-activated solution in vivo successfully demonstrated the anti-tumoral effects on pancreatic cancer in mice [25].

The anti-cancer effects of CAP depend largely on RONS [26–32]. CAP generates a cocktail of RONS able to selectively kill cancer cells, without affecting healthy cells, through a mechanism of oxidative stress [24,26,27,31]. However, oxidative stress not only exhibits an anti-tumoral effect but may also display a pro-tumoral effect, which depends on the tumor type and the tumor environment [33]. Regarding bone, oxidative stress plays a crucial role in the maintenance of bone remodeling in a healthy fashion, but it is also involved in the development of bone malignancies and associated to poor prognosis [10,34–36]. Unfortunately, up to now, some studies have described the lethal effects of CAP in OS cells. However most of them have been performed in 2D cultures and lacking the 3D bone context [29,37–41]. Due to the divergent effect of the oxidative stress in the bone microenvironment, it is critical to determine whether the oxidative stress generated by CAP has a real beneficial outcome for treating OS patients in a relevant scenario. In general, the use of CAP in 3D models is poorly understood; only a few works describe the effects of direct CAP treatment using spheroids and collagen-based matrices. These studies demonstrated a low diffusion of RONS into 3D models and a loss of CAP efficiency in 3D compared to 2D [28,42–44].

The aim of this study is to develop a suitable 3D engineered OS tumor model which allows to record for the first time the effects of plasma-activated Ringer’s (PAR) on a relevant tumor microenvironment. It is also our aim to take advantage of this 3D model to investigate potential remaining cancer stem-like cells of osteosarcoma after the PAR treatment.

2. Materials and methods

2.1. Synthesis and characterization of hydroxyapatite nanoparticles (nHA)

The procedure followed to synthetize nHA is fully described by Z. Zhao et al. [45] Briefly, a solution of 0.2 M H₃PO₄ (purity 85 wt %, Panreac) was added dropwise into 100 mL of 0.334 M Ca(OH)₂ (purity 96 wt%, Fluka) at a constant rate of 1 mL/min. When the pH reached 8, the reaction was stopped, and the suspension was left stirring 20–30 min. The following day, the suspension was rinsed with MilliQ water, centrifuged and lyophilized to obtain hydroxyapatite-nanoparticle powder.

The crystallinity and composition of the nanoparticles was assessed by X-Ray powder Diffraction (XRD) (D8 ADVANCE Twin, Bruker). Cu Kα radiation was used over a 2θ range of 20 – 50°. Scan step was set to 0.02, and a counting time of 2 s per point at 40 kV and 40 mA, and crystal morphology was assessed using a field emission Scanning Electron Microscopy (SEM, JEOL JSM-7001F). Prior to observation, the nHA were sputtered with carbon (EMITECH K950X Turbo Evaporator, Quorum Technologies Ltd., UK) to avoid charging effects.

2.2. Sponge-like Col1/nHA scaffold preparation

For the preparation of the Col1/nHA scaffolds, 6.3 mL of a solution of 4 mg/mL collagen (OptiCol™ Rat Type I Acid Soluble Collagen, Collagen Guidance System Ltd, UK) was mixed with 0.7 mL suspension of 50 mg/mL of nHA. After complete homogenization, the mixture was transferred to several molds so that 0.2 mL of the mixture was introduced in each mold. Then, the samples were frozen at –20 °C and lyophilized (Telstar Cryodos) at 0.010mBar and –75 °C overnight in order to obtain sponge-like scaffolds. In this particular case, the molds used were 1.5 mL Eppendorf’s caps. The dried sponge-like scaffolds were crosslinked by immersing them in a solution of 70% (v/v) ethanol containing 32.34 mM (3-Dimethylaminopropyl) -n-ethylcarbodiimide hydrochloride (EDC) (C₈H₁₇N₄ . HCl, CAS: 25952-53-8, purity ≥ 98.0%, Sigma-Aldrich®) and 5.91 mM of n-hydroxysuccinimide (NHS) (C₆H₇NO₃, CAS: 6066-82-6, purity 98%, Sigma-Aldrich®). The crosslinking solution was removed after 4 h immersion at room temperature and the scaffolds were rinsed with deionized water four times to eliminate any residual chemicals. Then, the scaffolds were frozen, lyophilized overnight and stored in a desiccator.

2.3. Biomatериал characterization

2.3.1. Scaffold porosity and density

Mercury Intrusion Porosimetry (MIP) was performed using an Autopore IV 9500 porosimeter (Micromeritics Instrument Corporation, USA). Mercury intrusion-extrusion curves were recorded from 0.49 to 29996 psia in order to obtain pore entrance size distribution and bulk/ envelope density of Col1/nHA scaffolds. For this measurement, four scaffolds were introduced in the specimen holder. The specific surface area (SSA) of the Col1/nHA scaffolds was measured by He pycnometry (AccuPyc 1330, Micromeritics Instrument Corporation, USA). Six different specimens were measured three times and the mean value and standard deviation was obtained.

The total porosity was calculated using the following equation:

$$P_{\text{TOTAL}} = \left(1 - \frac{\rho_{\text{envelope}}}{\rho_{\text{skeletal}}} \right) \times 100$$  \hspace{1cm} (1)

The envelope density ($\rho_{\text{envelope}}$) was obtained by MIP (Micromeritics Instrument Corporation, USA) analysis, measuring at 0.49 psia. The
skeletal density \( (\rho_{\text{skeletal}}) \) was determined by Helium pycnometry (AccuPyc 1330, Micromeritics Instrument Corporation, USA).

2.3.2. Scaffold morphology and atomic constitution

After lyophilization, the morphology of the scaffold was investigated by Scanning Electron Microscopy (SEM) (Phenom XL Desktop SEM, PhenomWorld) operating at 10 kV. The samples were previously sputtered with carbon to avoid charging effects and images at different levels of magnification were acquired. SEM microscope was coupled with an energy dispersive X-ray spectrometer (EDS), that was used to test the surface atomic constitution of the scaffold. The structure of the Col1/\( nHA \) scaffold was observed by X-Ray Microcomputed Tomography (\( \mu CT \)) (SkyScan 1272, Bruker microCT, Kontich, Belgium). The scanning was conducted at 10 \( \mu \)m resolution, 60 kV and 166 \( \mu A \), using an Al filter. Rotational step was set to 0.2° over an angle of 360°.

2.3.3. Scaffold mechanical properties

Stress-strain curves of the scaffolds were obtained using a Discovery hybrid rheometer (Discovery HR-2, TA Instruments), equipped with a 0.5 mN resolution load cell. A uniaxial load at a constant strain rate of 0.1 mm/s was applied on the samples (diameter: 6 mm and thickness: 2 mm). The compressive modulus was defined as the slope of the linear fit between 2% and 10% strain \( (n = 3) \). The compression test was done to non-crosslinked as well as crosslinked Coll/\( nHA \) scaffolds.

2.3.4. Swelling

A gravimetric method was conducted to determine the water absorption/uptake of the composite scaffold. Four samples of 6 mm diameter and 2 mm thickness were used. Firstly, completely dry samples were weighted and then immersed in 10 mL of Ringer’s saline at 37 °C. The scaffold weight was recorded at different times until the equilibrium stage was reached. Consequently, the swelling percentage was calculated as:

\[
\text{swelling} = \frac{w_s - w_i}{w_d} \times 100
\]  

(2)

where \( w_s \) is the dry weight of the scaffold and \( w_i \) means the weight of the scaffold with Ringer’s absorbed.

2.3.5. Degradation assay

To check the stability of the scaffold in Ringer’s saline media, dried scaffolds \( (n = 4) \) were immersed in Ringer’s saline at 37 °C for 15 days. Then, the samples were submerged in deionized water for 2 h to remove the salts adsorbed inside the scaffold. The bath was gently shaken by means of a shaker and the deionized water was changed every 30 min. Final weight of the dried scaffold \( (w_d) \) was measured and the mass loss percentage \( (m_l) \) was calculated as:

\[
m_l = \frac{w_d - w_i}{w_d} \times 100
\]  

(3)

where \( w_d \) is the initial dry weight of the scaffold.

2.4. Cell culture

An Osteosarcoma cell line MG-63 (CRL-1427™, ATCC collection) was employed. Given the complexity in the development, characterization of the scaffold, the validation of the 3D tumor model, and that the main aim of the paper was testing for the first time reactive species in plasma-activated saline in the novel tumor model, the study was restricted to this cell line. Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) ( Gibco™), supplemented with 10% fetal bovine serum (FBS) ( Gibco™), 2 mM L-glutamine ( Gibco™), 100 units/mL penicillin ( Gibco™) and 100 \( \mu \)g/mL streptomycin ( Gibco™). Cells were maintained in 3D culture prior to cell seeding.

The 3D-culture was performed as follows: Before 3D seeding, 30 \( \mu L \) of complete DMEM was added to the liofilised dry scaffold to hydrate it in cell culture conditions for 4 h. Following immersion in DMEM, the scaffolds were seeded with cell suspensions (300.000 cells in 20 \( \mu L \) DMEM/scaffold) and maintained at 37 °C, 95% humidity and 5% \( \text{CO}_2 \) during 1 h to increase cell attachment (Experimental scheme in Fig. 1). After that, the scaffolds were transferred to non-adherent 6-well plates with 6 mL of DMEM which was replaced every 3 days.

2.5. Cell proliferation in 3D cultures

Cell proliferation was determined using Quant-IT™ PicoGreen® dsDNA Kit (Invitrogen, #P11496) at day 0 (4 h post-seeding) 3, 6 and 9 days \( (n = 4) \). Scaffolds were collected in 1.5 mL Eppendorf tubes and frozen at –80 °C immediately after harvesting. To extract DNA from frozen samples, we used a 1 mL of digestion solution composed to each seeded scaffold. Digestion solution: Papain 125 \( \mu \)g/mL (Sigma-Aldrich, #P4762), l-Cysteine 0.242 mg/mL (Sigma-Aldrich, #C1276) and EDTA 0.33 M (Sigma-Aldrich, #E6511) in sterile DPBS (GibcoTM, #14190). Samples were digested in a 1.5 mL eppendorf tube at 65 °C overnight. The following day, samples were completely disaggregated by vortexing, and centrifuged 1 min. Briefly, DNA samples were diluted 1:10 in 1x TE buffer. 100 \( \mu \)L of diluted sample were incubated with 100 \( \mu \)L PicoGreen® reagent (1:1) and incubated 5 min using dark flat-bottomed 96-well plate. The fluorescence was read at excitation 485 nm and emission 525 nm and was compared to a DNA standard curve.

2.6. Gene expression analysis

Total RNA was extracted from whole 3D cultures using TRIzol™ Reagent (Invitrogen) following the manufacturer’s protocol. The samples were mechanically disintegrated in DPBS before incubation during 10 min in 1 mL of TRIzol™. RNA isolation was performed by chloroform/isopropanol method. RNA was precipitated overnight at 4 °C using ethanol and resuspended in 50 \( \mu L \) RNAase free water. To complete RNA isolation, RNA samples were purified using RNA RNeasy Mini Kit columns (Qiagen, # 74104). cDNA synthesis was performed using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase (Thermo Scientific, #K1671), 1 \( \mu g \) mRNA was loaded. The gene expression was assessed by using SYBR® Green PCR Master Mix (Applied Biosystems™). GAPDH was used as a housekeeping gene. Primer sequences used are shown in Supplementary Information Table S1.

Overexpression of selected genes in Osteosarcoma tumors and MG-63 osteosarcoma cell line were compared using the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) The R2 platform is an online genomics analysis tool that can analyze a large collection of public data. We selected bone-related genes and MMP9 related to cancer progression as genes of interest to generate a MegaSampler using the following datasets:

- Tumor Osteosarcoma: Kobayashi: GEO ID: gse14827. Dataset date 2010-02-01. Number of samples 27. GeneChip Human Genome U133 Plus 2.0 array \[36\].
- MG-63 cell line: Versteege dataset. R2 internal identifier: psavgreen_itcincinnatim86_u133p32. Number of samples: 86. Dataset Date: 2000-01-01. Platform: GeneChip Human Genome U133 Plus 2.0 arrays.

2.7. Fluorescence imaging and immunofluorescence

Hydroxyapatite nanoparticles (\( \text{nHA} \)) were labeled with fluorescein isothiocyanate (FITC) as explained in Ref. [47]. Briefly, 50 mg of HA-NP were incubated in a mixture of 10 mL of (3-aminopropyl) triethoxysilane (Sigma-Aldrich #440140) and 50 mL of anhydrous ethanol for 3 h at 74 °C in constant agitation. Then, 25 g of FITC was added into the suspension and allowed to react for 6 h. \( \text{nHA} \) were washed twice with anhydrous ethanol and ultrapurified water. Finally, FITC-labeled nanoparticles were obtained by freezing and lyophilizing.
For immunofluorescence staining, MG63 cells were incubated into the Scaffolds for 24 h. After incubation, scaffolds were transferred to DPBS, and washed 3 times with DPBS, cells were fixed with 1 mL 4% of paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. After washing twice with DPBS, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in DPBS for 5 min at 4°C, washed 30 min in DPBS, and blocked with SuperBlock™ T20 (PBS) Blocking Buffer (Thermo Scientific, #37516)) in agitation during 1 h. Then, cells were washed and incubated with Alexa Fluor™ 546 Phalloidin (1:300 in SuperBlock™ T20) during 1 h in the dark. Cells were washed 30 min in agitation with DPBS and the whole scaffolds were mounted with Fluoroshield Mounting Medium with DAPI (Abcam, #ab104139). Images were captured using Zeiss laser scanning microscope using z-stack and tiles region over the whole scaffold. Immunofluorescence images were taken at 10X. Scale bar: 1000 μm.

2.8. Histology and immunohistochemistry (IHC)

Tumor tissue constructs were fixed in 4% PFA for 24 h and then samples were dehydrated in graded ethanol washes and embedded in paraffin. Serial sections (5 μm thick) were prepared for histology and stained with hematoxylin and eosin (H/E).

Immunohistochemistry staining was performed using primary antibody specific to Ki67 (dilution 1:100, Abcam, ab15580), and developed using the Vector Elite ABC kit (Vector Laboratories), following manufacturer instructions. Briefly, sections were blocked with serum for 30 min and incubated with the primary antibody overnight at 4°C. After washing with PBS, samples were incubated with secondary antibodies and developed (Vector Laboratories). Negative controls were prepared by omitting the primary antibody step.

2.9. Plasma Activated Ringer’s (PAR)

We employed an atmospheric pressure plasma jet (kINPen® IND, Neoplas tools GmbH, Greifswald) to obtain PAR. The device consists of a hand-held unit that produces a plasma discharge at atmospheric pressure, using Argon as gas to generate the discharge [48]. To obtain the plasma-activated Ringer’s saline (PAR), 1 mL of sterile Ringer’s saline solution (8.6 g/L NaCl, 0.3 g/L KCl and 0.33 g/L CaCl₂·2H₂O) was placed in 24 well-plate under plasma jet at room temperature. PAR was activated using the following CAP parameters: Argon flow of 3 L/min at a distance of 10 mm from the jet nozzle to the surface of the liquid, using treatment times from 30 to 240 s 10% of FBS was added to PAR immediately after treatments to maintain physiological pH range [49].

We studied the concentration of H₂O₂ and NO₂⁻ after the plasma treatments employing the protocol previously reported, based on Amplex Red and on Griess Reagent, respectively [29,49].

2.10. Effects of PAR on 2D and 3D cultures

To assay the effects of PAR on cell viability of 2D and 3D cultures, tumor tissue constructs at Day 6 were washed once with DPBS and
exposed to 1 mL of PAR + 10% in a 24-well plate. Each culture was incubated during 2 h with PAR + 10% FBS for each condition in quadruplicate. Untreated PAR was used as control. Afterwards, 3D Culture + PAR was transferred to a non-adherent 6-well plate adding 3 mL of complete DMEM, thus exposing cells to ¼ diluted PAR during 24, 48 or 72 h. To compare PAR effects to 2D culture, 1 × 10⁶ cells were cultured in T-75 flask, and were treated with 8 mL of PAR (¼ diluted). Cell cytotoxicity was assessed using Picogreen Assay, and LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen, #L3224) following manufacturer’s protocol and IQH previously described.

To test the ability of PAR to eliminate Cancer Stem Cell sub-populations, tumor tissue constructs 72 h after PAR treatment were recovered for each condition (n = 3). Scaffolds were disaggregated mechanically and trypsinized to recover cells and were resuspended in 1 mL DPBS. Live cells were counted by Trypan Blue method and plated 1500 cell/well in ultra-low attachment 6-well plate into sarcospheres floating culture (n = 6), allowing growth during 10 days, following the method described in Refs. [50,51].

2.11. Statistical analysis

All data are presented as means ± SD. Statistical analysis of the data was performed using a Student’s t-test. p-values < 0.05 were considered statistically significant.

2.12. Graphics

Some of the graphics were made with BioRender.com.

3. Results

3.1. Fabrication and characterization of the bone-like scaffold (Col1/nHA)

A novel 3D scaffold was fabricated as a biomimetic niche for osteosarcoma cells to investigate the effect of CAP-activated liquids on a 3D cancer microenvironment. The composite biomaterial was obtained from the blend of Collagen Type 1 (Col1) and hydroxyapatite nanoparticles (nHA) to mimic both the organic and inorganic phases present in bone. The biomaterial was fabricated by the freeze-drying technique.

![Fig. 2. Microstructural characterization of the Col1/nHA scaffolds by μCT and SEM. (a) μCT cross-section image showing a homogenously distributed porosity within the whole section of the scaffold. (b) Scanning electron micrograph showing the porous structure of the scaffold. (c) SEM image of a collagen scaffold without the addition of nHA particles. (d) SEM image revealing the presence nHA particles and their uniform distribution throughout the scaffold.](image-url)
according to the experimental scheme of Fig. 1, yielding a final composition of \( \sim 58\% \text{ wt of nHA.} \)

The material developed is a highly porous scaffold as shown by \( \mu \text{CT} \) and SEM images (Fig. 2a and b), Supplementary Fig. S1, with macro-porosity following a unimodal curve centered at 60 \( \mu \text{m} \) (Fig. 3a), and with a high porosity close to 86\% and high SSA of 22.38 \( \text{m}^2/\text{g} \) (Table 1). Col1/nHA shows a physisorption isotherm identified as type IIb that has an H3 type hysteresis loop (Fig. 3b). This corresponds to adsorbents having non-rigid aggregates of nanoparticles, in agreement with the SEM images, which showed the presence of nanoparticles uniformly distributed within the Col1 (Fig. 2d). While the material is compressible with a low Young’s modulus around 2.5 kPa, the crosslinking of collagen 1 improved its mechanical properties, as shown in the stress-strain curve (Fig. 3c). The composite material is hydrophilic, with a high capacity to quickly absorb water (Swelling ratio = 2500\%, Fig. 3d). It is also highly stable due to the crosslinking step with EDC/NHS, with just a weight loss of 10.82\% \( \pm 2.86\% \) in two weeks (Table 1).

### 3.2. Col1/nHA scaffold promotes OS cells proliferation and osteomimicry in vitro

Three hundred thousand MG-63 osteosarcoma cells were seeded into Col1/nHA scaffolds and cultured for 9 days. nHA were labeled with FITC (nHA-FITC). Immunofluorescence imaging confirmed the uniform distribution of the nanoparticles within the scaffold (Fig. 4a). MG-63 cells were distributed evenly 4 h post-seeding as imaged by nuclei (blue, DAPI) and actin staining (red) (Fig. 4a). Cell proliferation in the Col1/nHA scaffolds showed a progressive increase in cell proliferation of 8-fold on day 9 (Fig. 4b). RT-qPCR was used to investigate the ability of Col1-HA material to induce genes expressed by bone cells and OS cells, a phenomenon known as osteomimicry (Fig. 4c). Transcriptional analysis showed that the expression levels of all genes studied in MG-63 cultured within the 3D biomaterial were significantly up-regulated in a time-dependent manner, showing maximum levels of expression at day 9. Gene expression increased 20-fold for Fibronectin (FN-1), 12-fold for Focal Adhesion Kinase FAK (PTK2) and the Metalloproteases (MMP2 and MMP9) displayed around 6-fold change (Fig. 4c). Together these data confirm that OS seeded in the Col1/nHA scaffolds are able to increase cell communication and interaction with the extracellular matrix.

| Property                          | Value               |
|----------------------------------|---------------------|
| Skeletal density (g/cm\(^3\))    | 0.274 \( \pm 0.017 \) g/cm\(^3\) |
| Porosity (%)                     | 85.95\%             |
| SSA (m\(^2\)/g)                  | 22.38 m\(^2\)/g     |
| Weight loss after 15 days (%)    | 10.8 \( \pm 2.9\% \) |
| Young’s modulus after crosslinking (kPa) | 2.5 \( \pm 0.6 \) kPa |

Table 1: Physical characterization of the Col1/nHA scaffolds.
Osteocalcin (BGLAP) gene, 5-fold for the Alkaline Phosphatase (ALPL), 30-fold for BMP2 and 10-fold for RUNX2, reflecting that the human OS cells activate the osteomimicry response. To further confirm the results, we performed a genomic analysis of OS microarray datasets that revealed higher RUNX2, BGLAP, ALPL, BMP2, and MMP9 mRNA levels in OS patients than MG-63 cultured in 2D (Fig. S2). Thus, we concluded that the Col1/nHA scaffold induced a similar gene expression signature in OS cells cultured in vitro than the one observed in native tumors from patients.

3.3. Effects of Plasma Activated Ringer’s (PAR) in human OS 2D cultures and in the 3D tumor model

In order to compare the effects of PAR, similar number of cells was seeded both in 2D and 3D cultures. For this analysis, the tumor tissue constructs at day 6 were used. In both cases, cells were exposed to 1 mL of PAR+10%FBS during 2 h, and then maintained during 72 h in PAR diluted in DMEM to ¼. Cell proliferation at day 9 (Fig. 5a) revealed interesting effects; in 2D culture, PAR induced a decrease in cell proliferation in a dose-response manner, reducing 50% of cell population when treated with 30 s PAR but being totally cytotoxic when the culture is treated with 120 s PAR. The same treatment showed completely distinct behavior in the 3D tumor model; PAR increased cell proliferation in a dose response manner up to 2.5-fold, with a clear threshold between stimulation of cell proliferation and cytotoxicity. The 240 s of plasma treatment in PAR was completely cytotoxic for the 3D cultures (Fig. 5a and b). The concentration of H₂O₂ and of NO₂ was quantified in 1 mL of Ringer’s Solution treated with the kINPen® IND plasma jet, confirming that the generation of these reactive species in PAR after plasma treatment is time dependent (Fig. 5c). As expected, significantly lower concentrations of NO₂ (from 2 to 44 μM) were detected in PAR with respect to those of H₂O₂ (from 40 to 550 μM) (Fig. 5c). RONS seem
to be key players in the cellular effects of PAR. Therefore, with the purpose of exploring the great differences observed between 2D and 3D cultures, stability and concentration of H₂O₂ and NO₂⁻ was monitored through different experimental stages, for a treatment time of 120 s. Peroxides and nitrates were quantified in Ringer’s (PAR), in PAR and 10% of FBS (PAR + FBS), in PAR + FBS in contact with the Col1/nHA scaffold (PAR + FBS + Sc) and also after the final addition of DMEM (PAR + FBS + Sc + DMEM) (Fig. 5 d,e).

First, it was found that after the addition of 10% FBS the concentration of peroxide was reduced from 300 to 280 μM only due to dilution. After 2 h of FBS addition, peroxydes decreased to 180, showing a higher aging effect compared to PAR alone. The presence of the scaffold (PAR + FBS + Sc) provoked a scavenging effect reducing the concentration of peroxydes down to 150 μM. Finally, the real dose received by the OS-2D & 3D cultures after the first 2 h is only 50 μM because of the dilution with DMEM. This dose tended to diminish in the next 72 h (Fig. 5d). The concentration of NO₂⁻, contrary to H₂O₂, increased slightly in presence of FBS and the Col1/nHA scaffold (Fig. 5e), indicating that the scaffold acts as scavenger of H₂O₂ only. These results demonstrate that the dose of H₂O₂ between 2D and 3D cultures is, essentially the same. The concentration of NO₂⁻ is stable up to 24 h, becoming undetectable at 72 h.

Haematoxylin/Eosin (H&E) and Ki-67 immunostaining allowed to evaluate histological effects of PAR in 3D-OS cultures after 72 h of treatment (Fig. 6a). A significant reduction of relative nuclei area (H&E staining) was observed to be induced by PAR with increasing plasma treatment times. However, a high number of Ki-67 stained were observed in both treatments independently of PAR treatment (Fig. 5b), with higher amount of Ki-67 in the surviving cells (Fig. 6a). We performed RT-qPCR in MG-63 cells seeded in the 3D scaffold, treated for 72 h with non-lethal doses of PAR 30, 60 and 120 s to analyze the expression of genes with well-known functions of cancer stem cell (CLSC) phenotype. The surviving fraction to PAR showed a significant induction of genes related to stemness and Cancer Stem Cell phenotype in OS, like SOX2, OCT3/4 and NANOG (Fig. 6b) and surprisingly the treatment times recorded an increase on cell proliferation in 3D cultures (Fig. 5a and b). To explore how PAR can affect CLSC subpopulations, we recovered live cells from 3D-OS cultures treated by PAR 120 and 240 s, and surviving cells were plated in CLSC conditions [50]. It was observed that PAR promoted an increase (2-fold) of the sarcospheres formed from 3D-OS cultures respect to the control (Fig. 6c), increasing both the number of sarcospheres and their size (Fig. 6d).

4. Discussion

Cold atmospheric plasma and plasma-activated liquids have been postulated as potential therapies against cancer. Most of the published studies rely on cancer cell monolayers, which lack the 3D tumor microenvironment and are not good predictors of the responses to therapies. Therefore, developing bona fide tumor models that include the
3D niche is a must for testing and validating the CAP therapy. In this work, we investigated the effect of CAP activated ringer’s saline on osteosarcoma cells cultured within a 3D bone-like biomaterial, that mimics the osteosarcoma microenvironment.

According to Monteiro et al. [18] an ideal bone scaffold should have micro and macroporosities (pore size ≤ 20 μm and 100 μm) with a controlled resorption rate. In this work, we designed that ideal bone scaffold as a niche for osteosarcoma cells using Col1 and HA nanoparticles. The Col-1/nHA biomaterial generated is a highly porous scaffold with a controlled diameter of 6.00 ± 0.02 mm and thickness 2.02 ± 0.03 mm (Fig. S1a). The hydroxyapatite nanoparticles (nHA) have been described in detail in earlier works [45]. These nHA have shown to be internalized when in suspension, but stabilization of a negative surface charge on the surface of the nHA prevents its internalization in MG63. Moreover, in our work, being incorporated in the composite, this internalization is prevented, and the particles are a suitable source of Ca²⁺ and PO₄³⁻ mimicking bone, which is also constituted of HA nanoparticles. Owing to the interconnected pore structure and SSA of the scaffold, MG-63 human osteosarcoma cells are capable to attach homogeneously 4 h after seeding (Fig. 4a), penetrate into the pore network and to proliferate until day 9 post-seeding (Figs. 4b, 5b). MG-63 cells cultured within the Col1/nHA scaffold show a time-dependent up-regulation of genes related to the bone pathogenesis, which indicates that the scaffold enhances cell growth and differentiation (Fig. 4). These data confirm not only that Col1/nHA composite scaffold is a biocompatible, but also a biomimetic material. We found that MG-63 cells in the 3D model increase the gene expression signature observed in patients but not in 2D monolayer cultures related to cell-matrix interaction, such as Fibronectin (FN1) involved in poor prognosis in osteosarcoma [52], FAK/MMPs axis related to cell invasion through extracellular matrix, focal Adhesion Kinase FAK (PTK2) involved in focal adhesion, invasion and metastases in many cancer types [53,54], including sarcomas [51,55], and matrix metalloproteases proteins 2 and 9 (MMP2 and MMP9) involved in tumor invasion and poor prognosis [56,57].

Evidence exists to support that a mesenchymal stem cell, as well as committed osteoblast precursors, are the cell of origin of osteosarcoma, and that bone environment is essential for osteosarcoma development and the process of osteomimicry (expression of bone-related genes) [8,9,58]. A number of studies use recombinant proteins, such as BMP2, to induce bone-related genes, bone differentiation and osteomimicry [9,58,59]. The scaffold developed here containing Col1/nHA favors osteomimicry of osteosarcoma cells in vitro by enhancing the expression of Osteocalcin (BGLAP), Alkaline Phosphatase (ALP), BMP-2 and RUNX2. These genes are expressed in the bone and are involved in pathways commonly associated with bone cancer malignant features [60–62]. We confirmed a time-dependent increase of expression of these genes in the 3D model, as observed in real tumors, but not in the 2D cultures (Fig. 4c; S2). Importantly, we did not supplement the medium with BMPs, so the biomaterial itself induces osteomimicry in MG-63 cells in a similar way that occurs in native tumors. Thus, our data point out the Col1/nHA composite as a valuable tool for developing biomimetic models of bone cancers and bone metastasis for anti-cancer therapies testing.

The lethality of plasma-treated liquids depends largely on the number of cells studied [63], and therefore we compared the effect of PAR in 3D cultures growth at day 6 to an approximately equivalent 1 × 10⁶ cells in 2D cultures to obtain comparable results (Fig. 5a). As expected, our results show opposite effects between 2D and 3D cultures; PAR reduces cell viability in a dose response manner in 2D cultures while it enhances proliferation in 3D cultures at the same increasing treatment times (Fig. 5a and b), except 240 s that eliminates cells both in 2D and 3D cultures. The gap between 2D and 3D cultures is clearly observed with PAR treated for 120 s, as in 2D cultures it shows 0% of viability, while in
3D cultures an increase of 2.5-fold proliferation was recorded (Fig. 5a).

The concentrations of H$_2$O$_2$ and NO$_2$ in the PAR at the different stages of treatment were related to its cytotoxic effects (Fig. 5c,d,e). H$_2$O$_2$ [63–66] and NO$_2$ [27,67] are two of the species most often measured in saline solutions treated by plasma [68,69], due to their long lifetime and availability of detection methods. As it has been shown in previous works through the addition of artificial H$_2$O$_2$ and NO$_2$ to Ringers Saline, these two species are not the sole responsible of the cold-plasma induced cytotoxicity [49]. In fact, many studies attribute the anti-carcinogenic effects of CAP to these and many other reactive species such as O$_2^*$, OH*, NO, O, NO$_3^-$, and ONOO$^-$ [70]. The addition of metabolites to PAR has been shown to increase their anti-tumor toxicity [25]. It has been shown that plasma-activated medium was able to eliminate tumor initiating cells in monolayer or in vivo by reducing ALDH high populations [71]. Other studies have shown that plasma-activated medium acts synergistically with metabolic inhibitors [72]. All these data demonstrate the importance of the composition of liquids treated by plasma in their reactivity.

Our results in 2D show that the accumulation of RONS in liquids depends on the treatment time (Fig. 5c), in line with previous results [29,41,49,73], maintaining high levels of H$_2$O$_2$ and lower concentration of NO$_2$ (Fig. 5c) in all treatment times studied. Only PAR activated during 240 s is completely effective in 2D and 3D cultures (Fig. 5a and b) corresponding to a high dose of H$_2$O$_2$ (400 μM) previously described as lethal to healthy and OS cells [29]. In order to explain the discrepancies between 2D and 3D cultures two things need to be taken into account: First, we reported previously that non-lethal doses of Plasma Activated Medium may stimulate the proliferation in human OS cells [29]; Second, other reports show the ability of scaffolds to act as RONS scavengers [74], as we verified here for peroxide, although to a very small extent (Fig. 5d).

In line with this data, here FBS, Scaffold and DMEM reduce the concentration of H$_2$O$_2$ in 3D cultures (Fig. 5d) and NO$_2$ (Fig. 5e), reducing the cytotoxic potential of PAR, but the concentrations in the situation in 2D without scaffold are very similar. On another hand, it is known that solid tumors have the ability of increase anti-ROS defenses by modulation of the Glutathione metabolism [75]. Here we found a similar trend in MG-63 cultured in Co1/HA scaffolds showing a culture-time dependent expression of genes related to detox-ROS pathways like Superoxide Dismutase (SOD1), Glutation-S-Transferase (GSTM1) and Glutation-S-Reductase (GSR) (Fig. S3). This data could explain in part the discrepancies between 2D and 3D cultures.

To gain insights about the effect of PAR on 3D tissues and cell resistance to therapies, we analyzed cell morphology in histological sections of our engineered tumor by Hematoxylin-Eosin (HE) and cell proliferation by immunohistochemical detection of Ki67. Our data show that PAR reduces the number of cells in a dose-response manner (Fig. 6a), and that PAR activated for 240 s was enough to eliminate 90% of proliferating cells in 3D cultures (Fig. 5a). However, the remaining cells show high levels of Ki67, which is related to poorly differentiated tumor cells and stemness [76], tumor relapse and tumor aggressiveness [76,77]. PAR activated during 120 s increase cell proliferation but shows the same trend for Ki67 expression (Fig. 5a). Together these data suggest that PAR enhances the stemness phenotype of osteosarcoma cells in the 3D model. This is a first-time evidence, very relevant for researchers in the field on how CAP-based therapies affect CSLC and tumor heterogeneity.

CSLC are subpopulations of tumor cells resistant to most current therapies like radiation and chemotherapy and they are responsible for tumor relapses and metastasis [8]. For this reason, to propose effective therapy against cancer is necessary to eliminate CSLC subpopulations. To confirm whether PAR induces the stemness phenotype of osteosarcoma cells, we analyzed the expression of genes expressed by CSLC [78–80]. We found for the first time that PAR increases the expression of SOX2, OCT3/4 and NANOG in 3D cultures in a dose-response manner independently of cell proliferation (Figs. 5 and 6b). PAR enhances the expression signature associated with CSLC phenotype in all conditions studied. The most enhanced effects were observed at the maximum lethal dose of PAR 240 s. Our data reveal that PAR favors the CSLC phenotype in OS independently of treatment time, and therefore the benefit of CAP treatment is not observed for OS in a 3D biomimetic model of the human disease. This contrasts to recent works where PAR has shown promising results in a murine OS tumor by organotypic cultures [49]. The behavior of CSLCs is tumor, microenvironment, and cell culture type dependent [8,81]. It is well documented that CSLCs are highly resistant to RONS in a similar way to healthy cells, showing i) the expression of enzymes to eliminate ROS, ii) quiescent state and iii) greater ability to repair DNA. Overall, the cytotoxic effects of RONS are highly related to disruption of mitochondrial metabolic but CSLC show a high plasticity to overcome mitochondrial damage by tuning metabolism [82–89]. Importantly, to the best of our knowledge, our work is the first report that shows the negative effects of the reactive species associated to plasma-treated liquids over CSLCs in a biomimetic 3D tissue-like environment.

5. Conclusion

Many studies reported that saline solutions activated by cold atmospheric plasma are promising therapies against a variety of cancers. However, very few works employ 3D environments to demonstrate how 3D cultures could affect the response of cell and cell populations to plasma-activated liquids. This is the first report that shows the effects of plasma-based therapies over cancer cell subpopulations in a 3D tissue-engineered model of human osteosarcoma. We fabricated the 3D model by developing a highly porous scaffold made of Co1 and HA nanoparticles. The material shows high stability, porosity and biocompatibility that allow osteosarcoma cells to grow and to acquire a similar gene expression profile to that observed in native tumors. Opposite effects of PAR were observed in 2D cultures and in the engineered tumor model, and can firstly be explained because 3D cell cultures are less sensitive to PAR induced cytotoxicity; this is due to the 3-dimensionality inducing the expression of a number of RONS-protective genes in MG-63 cells. Furthermore, 3D cultures facilitate the survival of OS sub-populations by enhancing the CSLC properties of OS cells, favoring cell proliferation and adaptation to oxidative stress induced by PAR treatment. This is a key finding that has not been described earlier and will deserve investigation in a wider range of cell lines and that should be taken into account in the design of future therapies with plasma activated liquids.

Author contributions

JT, AV and CC conceived the project and designed experiments; AV designed and fabricated the biomaterial; JT, AV and XS performed experiments and analyzed data; JT, AV, XS and CC wrote the manuscript. MPG contributed to the review of the manuscript; CC supervised the study & obtained funding.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2020.12.437.

Data availability

The data associated to this paper are available in: https://upcommo ns.ucp.edu/handle/2117/184325.

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