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Short communication

In vitro effects of ascorbic acid on viability and metabolism of patients' osteosarcoma stem cells

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ABSTRACT

Stagnation in novelties of osteosarcoma (OS) treatment indicates the need for new therapeutic methods. OS cancer stem cells (OS-CSC) are taught to have the ability to self-renew and develop mechanisms of anticancer drug resistance, and this is why it is difficult to eradicate them. Their metabolism has been recognized as a potential target of therapeutic action. Ascorbic acid (AA) is considered to act pro-oxidative against OS-CSC *in vitro* by oxidative effect and by inhibition of glycolysis. This study examined an *in vitro* impact of AA on OS-CSC metabolism isolated from patients' biopsies, with the aim of better understanding of OS-CSC metabolism and the action of AA on OS-CSC. OS-CSC were isolated using a sphere culture system and identified as stem cells using Hoechst 33342 exclusion assay. Determination of the dominant type of metabolism of OS-CSC, parental OS cells, human mesenchymal stem cells (hMSC) and U2OS OS lineage before and after AA treatment was done by Seahorse XF (Agilent). Cytotoxicity of high-dose AA was confirmed by the MTT test and was proven for all the examined cell types as well as HEK293. Seahorse technology showed that OS-CSC can potentially use both glycolysis and oxidative phosphorylation (OXPHOS), and can turn to glycolysis and slow metabolic potential in unfavorable conditions such as incubation in AA.

Keywords: osteosarcoma, cancer stem cells, tumor cell metabolism, ascorbic acid

Stagnation in therapeutic approaches to osteosarcoma (OS) treatment for the last 30 years indicates the need for new therapeutic methods (1). The tumor is composed of various cell subpopulations, among which OS cancer stem cells (OS-CSC) are considered to have the ability to self-renew and have developed mechanisms of anticancer drug resistance, thus being responsible for the disease relapse (2). Significance in prognostic and therapeutic implications of CSC is emphasized in a strong association between tumors

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with high CSC fractions and recurrence, poorer overall survival and higher incidence of metastasis (3). Given the mechanisms of resistance, it is difficult to eradicate them, so their metabolism has been recognized as a potential target of therapeutic action (4). Oxidative phosphorylation (OXPHOS) is a more efficient source of energy as it generates more ATP molecules than glycolysis, so in normoxic conditions, normal cells rely primarily on OXPHOS. Glycolysis is used by cancer cells often exposed to hypoxia, acidosis and lack of nutrients but they can engage glycolysis even in the presence of oxygen. This phenomenon is known as the Warburg effect (5). Upregulation of glycolytic enzymes and stimulation of glycolysis are also necessary for cell immortalization (6), glycolysis-driven induction of pluripotency and control of stemness characteristics (7). Increased mitochondrial cell function goes with higher rates of oxygen consumption and mitochondrial reactive oxygen species (ROS) production, enhanced mechanisms for resistance to DNA damage caused by oxidative stress (8), but also for CSC propagation (9). Some reports suggest that CSC preferentially use glycolysis even in normoxic conditions, other suggest a preference for OXPHOS (10). In addition, CSC may use other molecules for ATP and NADH generation, such as pyruvate, lactate, glutamine, glutamate, alanine, or ketone bodies, as well as aberrant metabolic pathways. In nutrient-poor states, they rely on autophagy (11). Metabolic phenotypes of CSC also depend on their location, such as actively growing tumor regions with adequate levels of oxygen, hypoxic areas and metastatic sites and on the crosstalk between CSC and cancer-associated stroma in the microenvironment (12).

According to previous *in vitro* studies, ascorbic acid (AA) induces apoptosis of OS-CSC cell lines by oxidative effect and by inhibition of glycolysis. This explains the ongoing interest in vitamin C in the fight against cancer. Vitamin C (L-ascorbic acid) is a reducing agent that is easily oxidized to dehydroascorbic acid (DHA). It is involved in a variety of oxidative mechanisms. Vitamin C is transported into cells by sodium-dependent ascorbic acid transporters, but it can enter in DHA form by glucose transporters. Following the transportation of DHA into cells, it is reduced to L-ascorbic acid using glutathione (GSH) thus changing the reduced glutathione ratio and causing the accumulation of ROS. It is taught that glycolysis can be altered by damaging glyceraldehyde 3-phosphate dehydrogenase (GAPDH) through vitamin C-mediated pro-oxidative effect (13). The physiological concentration of vitamin C in plasma is < 0.1 mmol L^{-1}. Vitamin C in high concentrations of 1–10 mmol L^{-1} (depending on cell lines) is taught to be toxic for cancer cells *in vitro* and harmless for healthy cells, so intravenous infusions of vitamin C are proposed for the eradication of CSC, especially if they are glycolytic (14).

When it comes to OS, there are not many reports on OS-CSC metabolism in the context of vitamin C treatment. The aim of this study is to examine an *in vitro* impact of AA on the viability and metabolism of OS-CSC isolated from tumor tissue samples obtained during diagnostic biopsy from children and adolescents, the population most affected by OS.

EXPERIMENTAL

Chemicals

Fetal bovine serum, KnockOut serum replacement, EGF and FGF were purchased from Gibco (USA). Penicillin/Streptomycin was obtained from Capricorn Scientific. Coon's

modified Ham's F12 medium, Dulbecco's Modified Eagle's Medium – high glucose, trypsin--EDTA, collagenase type II, Hoechst 33342 dye, L-ascorbic acid and methylcellulose (MC) were purchased from Sigma-Aldrich (USA). Dimethyl sulfoxide was obtained from Thermo Fisher Scientific (USA) and bicinchoninic acid from Santa Cruz Biotechnology (USA). Seahorse XF Cell Energy Phenotype Test Kit with Seahorse XF Base Medium that consists of pyruvate, glutamine, and glucose, and stressor mix oligomycin and FCCP (included in the kit) were purchased from Agilent Technologies (USA).

Parental OS cells isolation and sarcosphere formation

OS cells were isolated from patients' tumor tissue samples obtained during diagnostic biopsies. We consecutively obtained 3 samples, one from each patient, stored in a falcon tube with growth medium (GM, 10 % fetal bovine serum FBS (Gibco), 1 % Pen/Strep, in Coon's modified Ham's F12 medium) and sent to the laboratory where the tissue was cut into small pieces (0.5–1 mm) and transferred into collagenase medium (CM, 20 % FBS, 1 % Pen/Strep, 3.75 mg mL^{-1} collagenase type II in Coon's modified Ham's F12 medium). The cell suspension was transferred into a T-75 flask and incubated at 37 °C. After the cells were attached, the medium was changed and cells were cultured in a growth medium (GM, 10 % FBS, 1 % Pen/Strep in Coon's modified Ham's F12 medium). This primary OS cell culture (parental cells) was used for the sarcosphere assay. 3 mL of the trypsin-EDTA was added to dissociate the cell monolayer. Cells were counted using the hemocytometer.

Sarcosphere isolation

Methylcellulose (2 % in miliQ-H_2O) and sarcosphere growth medium (SGM, 20 % KnockOut serum replacement (Gibco), 1 % Pen/Strep, 0.01 % EGF, 0.01 % bFGF, in Coon's modified Ham's F12 medium) were prepared and combined in equal ratio to the concentration of 1 % MC. 4×10^4 cells/well were seeded in 6-well ultra-low attachment plates in 5 mL of MC-SGM. Growth factors were added two times a week and sarcosphere formation was observed by microscope and photographed. After 2–4 weeks, sarcospheres were isolated using 40 μm nylon cell strainers (Thermo Fisher Scientific) and they were seeded into a flask for adherent cell culture in stem cell growth medium (SCGM, 10 % FBS, 1 % Pen/Strep in Coon's modified Ham's F12 medium, 0.01 % bFGF). Under adherent conditions a cell monolayer was formed, representing the first generation of OS-CSC. The whole procedure was repeated to produce the second generation of OS-CSC, which were used for the following experiments.

Hoechst 33342 dye uptake assay

The second generation of OS-CSC isolated from the patients, parental cells, HEK293, hMSC and U2OS cell lines were seeded on 96-well plates at the cell density of 6×10^4 cells/well in the appropriate culture medium (Table I).

5 μg mL^{-1} of Hoechst 33342 dye in a total of 100 μL appropriate culture medium was added to each well. Control cells were left untreated. Fluorescence was measured on a GloMax microplate reader (Promega), (excitation $\lambda = 360$ nm, emission $\lambda = 450$ nm) and the

Table I. Cell types and belonging culture media used in the study

Cell type	Basic cell culture media
U2OS	10 % FBS, 1 % Penicillin/Streptomycin in DMEM – high glucose
HEK293	10 % FBS, 1 % Penicillin/Streptomycin DMEM – high glucose
hMSC	10 % FBS, 1 % Penicillin/Streptomycin DMEM – low glucose, 0.01 % bFGF
Primary osteosarcoma culture	growth medium (GM, 10 % FBS), 1 % Penicillin/Streptomycin in Coon's modified Ham's F12 medium
Osteosarcoma stem cell line – 1st generation	stem cell growth medium (SCGM, 10 % FBS, 1 % Penicillin/Streptomycin in Coon's modified Ham's F12 medium, 0.01 % bFGF
Osteosarcoma stem cell line – 2nd generation	stem cell growth medium (SCGM, 10 % FBS, 1 % Penicillin/Streptomycin in Coon's modified Ham's F12 medium, 0.01 % bFGF

results were analyzed using the R programming language. Since high expression of ABC transporters is a characteristic of stem cells, lower fluorescence assumes a higher percentage of stem-like cells.

MTT viability assay

The second generation of OS-CSC from the patients, parental cells, HEK293, hMSC and U2OS cell lines were seeded on 96-well plates at the density of 6×10^4 cells/well in the appropriate growth medium (Table I) and were allowed to adhere overnight. The next day, cells were treated in triplicates with AA in concentrations range 1 to 30 mmol L^{-1}. Fresh medium was added to negative control cells. After 24 h, treatment was aspirated and cells were washed 3 times with PBS. Cells were then incubated with MTT solubilized in an appropriate culture medium at a concentration of 0.5 mg mL^{-1} for 4 h. To dissolve formazan crystals, 170 μL of dimethyl sulfoxide (DMSO, Roth) was added to each well. After dissolution into formazan, the intensity of purple color in each well was measured on a GloMax microplate reader (Promega, USA), λ = 560 nm. IC_{50} (inhibition concentration where 50 % of the cells are dead) was calculated. A predictive modelling technique built in the extension package drc for the statistical environment R is used to construct dose-response models. The independent variable is referred to as the AA concentration, whilst the dependent variable is referred to as response *i.e.*, cell viability.

Bioenergetic profiling using Seahorse XFe24

Cells were plated 48 h before the assay on 24-well plates at the density of 6×10^4 cells/well in 100 μL of appropriate culture medium (Table I). 24 h before measuring, cells were exposed to 10 mmol L^{-1} AA, whereas the controls were left untreated. Seahorse XF Cell Energy Phenotype Test Kit was used.

The media were changed to assay medium (Seahorse XF Base Medium with 1 mmol L^{-1} pyruvate, 2 mmol L^{-1} glutamine and 10 mmol L^{-1} glucose) to the final volume of 500 μL. Before the assay, plates with cells and stressor mix (included in the kit) were prepared by solubilizing oligomycin and FCCP in the assay medium to the final concentration of

100 mmol L^{-1} and combined in appropriate amounts to create one 10 × solutions. Seahorse XF Cell Energy Phenotype assay was run following the manufacturer's instructions. Normalization of XF assay was applied on total cellular protein level measured by bicinchoninic acid assay (BCA). The assay results were analyzed using Wave Desktop software and Phenotype Test to study the effect of AA on cell metabolism.

Seahorse XF measures glycolysis and mitochondrial respiration. Glycolysis is expressed as the extracellular acidification rate (ECAR) and represents a readout of media pH value reflecting lactic acid and bicarbonate accumulation. Mitochondrial respiration is expressed as oxygen consumption rate (OCR) which is determined by extracellular oxygen level. Metabolic potential denotes the cell response to induced energy demand. We measured ECAR and OCR under baseline and stressed conditions for untreated cells and for cells incubated in 10 mmol L^{-1} AA at 37 °C and 5 % CO_2 for 24 hours. First, we measured the baseline OCR and ECAR, then the final values were measured after a simultaneous stressor mix adding to each well. Key parameters of cell energy metabolism revealed are: baseline phenotype, stressed phenotype and metabolic potential for treated cells, and all compared to the cells not treated with AA. The obtained data were normalized to post-assay protein harvest. Highly aerobic cells are susceptible to CO_2 production when stressed which can contribute to ECAR and over-report of glycolytic contribution to metabolic potential. Cells with a baseline OCR/ECAR ratio > 4 are identified as the cells that produce CO_2 which makes a negligible contribution to ECAR. On the other hand, the stressed ECAR value of cells with a baseline OCR/ECAR ratio < 4 could include both glycolysis and mitochondrial activity.

Statistical analysis

The extension package drc for the statistical environment R was used to carry out statistical analysis. Seahorse Wave Desktop Software (Agilent) was used for Seahorse results analysis. Experimental values were given as mean standard deviation.

Ethical statement

The research was approved by the ethics committee of Children's Hospital Zagreb.

RESULTS AND DISCUSSION

Stemness assessment

After 2–4 weeks, sarcospheres were isolated (Fig. 1).

The highest fluorescence intensity (the weakest dye efflux) was observed in U2OS cells that were used as the negative control in each of the experiments. hMSC showed low fluorescence. Low fluorescence was also measured in OS-CSC lines derived from all three samples, resembling the hMSC pattern.

Ascorbic acid effect on cell viability (MTT assay)

The percentage of live HEK293, hMSC, and U2OS to AA concentration is shown in Fig. 2a, whereas the response to different AA concentrations for the same samples is repre-

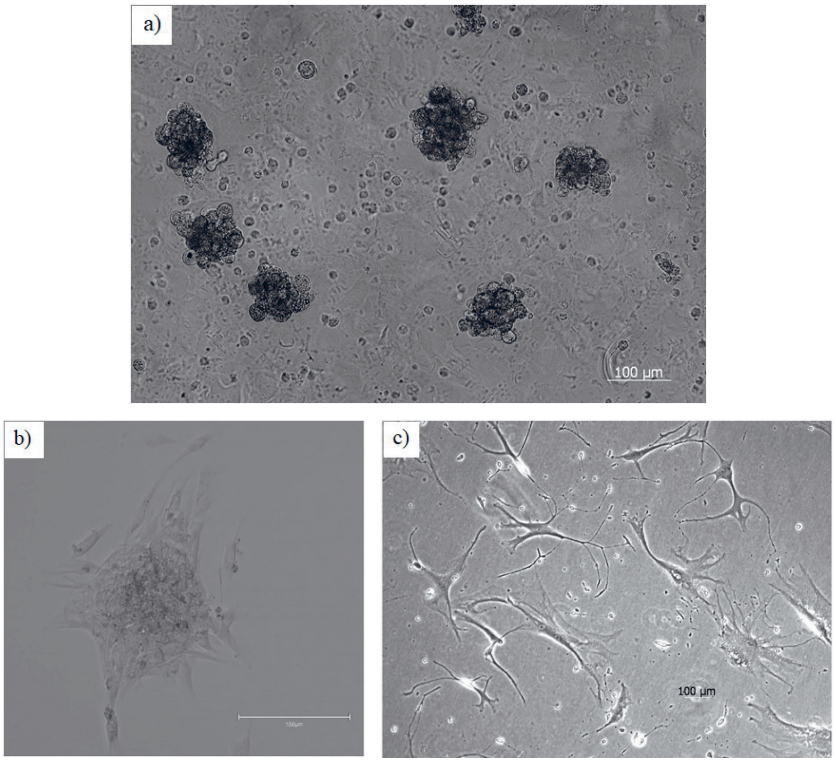

Fig. 1. Culturing of osteosarcoma stem sphere-forming cells. a) Non-adherent conditions supported the growth of osteosarcoma stem cells (OS-CSC) and the cells proliferated forming sarcospheres; b) after approx. 30 days, sarcospheres were isolated; c) exposed to adherent conditions where they attached and started forming a monolayer.

sented by the dose-response curves in Fig. 2b. Percentage of live HEK293 cells remained around 100 % until the AA concentration reached 10 mmol L^{-1}. For the next 2 concentrations, cell viability decreased by 10–20 %. The dose-response curve shows a mild decline between 15 and 25 mmol L^{-1} AA with the IC_{50} value being around 19 mmol L^{-1} AA. For the last 2 concentrations tested, the percentage of live cells was approximately zero.

hMSC viability decreased already after 5 mmol L^{-1} AA and varied around 50 % for the concentrations between 10 and 25 mmol L^{-1} AA until it dropped to almost zero when treated with the highest concentration. This cell line is the only one that does not show an expected S-shaped dose-response curve and instead assumes a sort of negative linear connection between viability and concentration. IC_{50} is estimated at around 18 mmol L^{-1}.

U2OS cells had almost constant viability of approximately 80 % until 20 mmol L^{-1} AA which drastically dropped as the concentration increased to 30 mmol L^{-1}. IC_{50} can be read out at 25.6 mmol L^{-1} – the highest value among the 3 control cell types.

All parental OS cells and OS-CSC samples derived from patients' tumors were grouped as parental *i.e.*, CSC, respectively. The percentage of live parental OS cells and

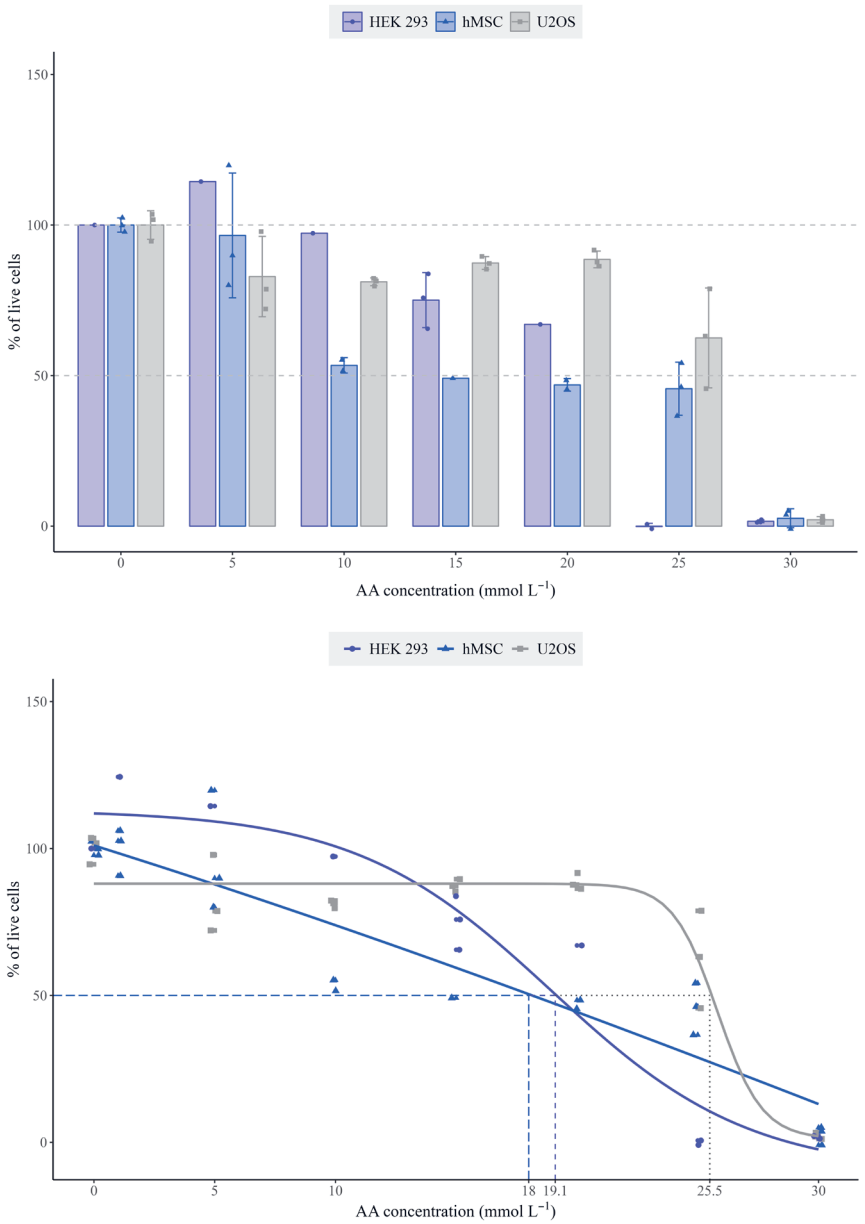

Fig. 2. Ascorbic acid effect on HEK293, hMSC and U2OS viability. a) Cell viability is expressed as a mean percentage of live cells (± standard deviation) as measured for 7 different ascorbic acid concentrations by MTT assay and as predicted by regression analysis; b) the IC_{50} value for each cell type is indicated. HEK293 – human embryonic kidney 293 cells, hMSC – human mesenchymal stem cells, U2OS – osteosarcoma cell line.

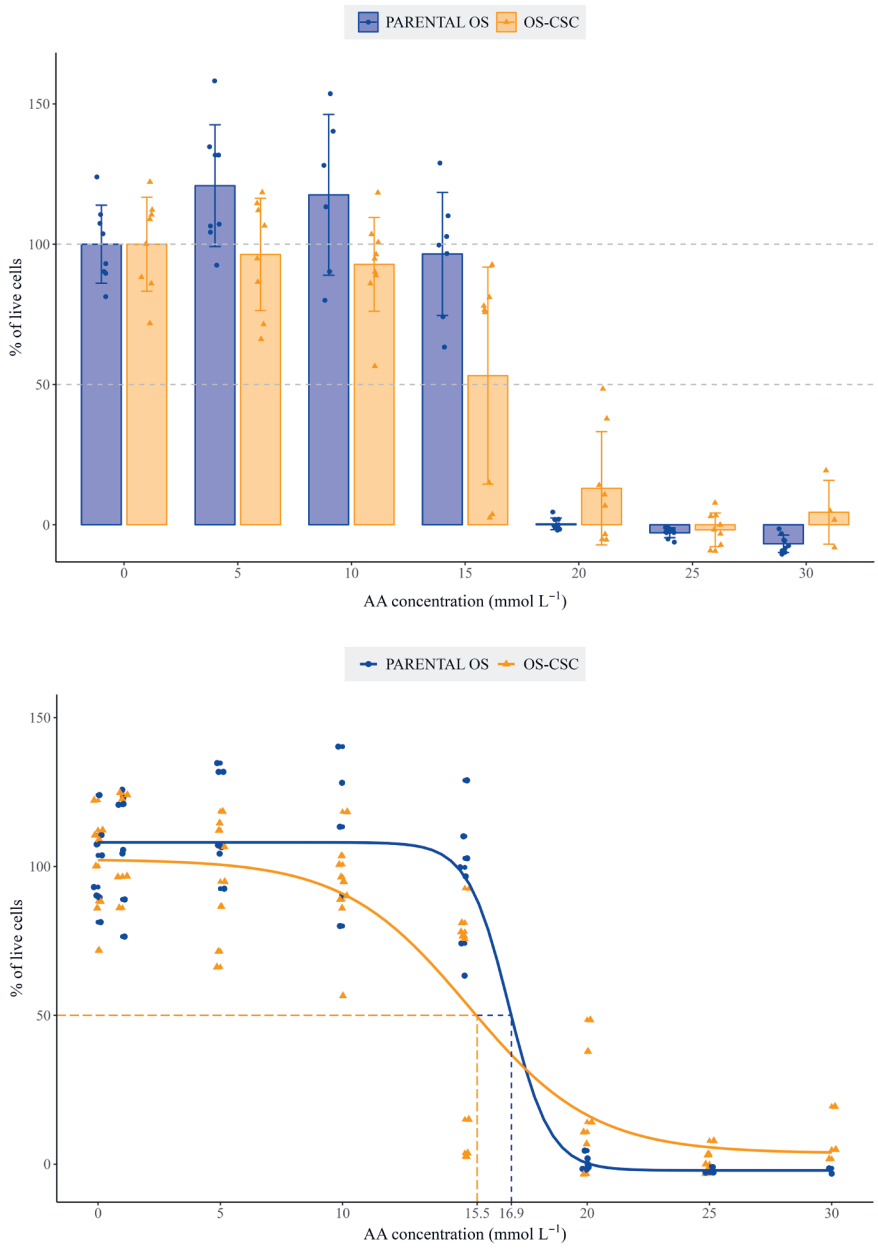

Fig. 3. Ascorbic acid effect on osteosarcoma stem cell viability. a) Cell viability is expressed as a mean percentage of live cells ± SD as measured for 7 different ascorbic acid concentrations by MTT assay and as predicted by regression analysis; b) the IC_{50} value for each cell type is indicated. Parental OS – parental OS cells, OS-CSC – osteosarcoma stem cells.

CSC relative to AA concentration is shown in Fig. 3a, while the response to different AA concentrations for the same samples is represented by the dose-response curves in Fig. 3b. When treated with AA, for parental OS samples cell viability was close to 100 % until the concentration of 15 mmol L^{-1}. A notable decrease is observed towards the next concentration, while the IC_{50} value was reached in between, at 17.1 mmol L^{-1}.

When treated with AA, for OS-CSC samples, the percentage of viability started to decrease at 5 mmol L^{-1}. The sharpest decrease was observed between 10 and 20 mmol L^{-1}, with the IC_{50} value of 15.5 mmol L^{-1}. From 20 to 30 mmol L^{-1} almost no cells remained alive for either of the two groups.

Ascorbic acid effect on cell metabolism

Generated phenotype maps are shown in Fig. 4. hMSC and U2OS cell lines were used as the control cells, while parental cells and OS-CSC derived from the patients' tumors were two experimental groups. In both baseline and stressed conditions, hMSC exhibited higher ECAR and lower OCR values for the cells incubated with 10 mmol L^{-1} AA compared to untreated. When treated with AA, U2OS cells expressed higher ECAR values in baseline and stressed conditions. On the other hand, OCR values for this cell type were elevated for the cells that were not exposed to AA. Parental cells phenotype map displays a decrease in stressed OCR and an increase in stressed ECAR values in treated compared to the untreated cells. Baseline values were roughly unchanged for treated *vs.* untreated cells.

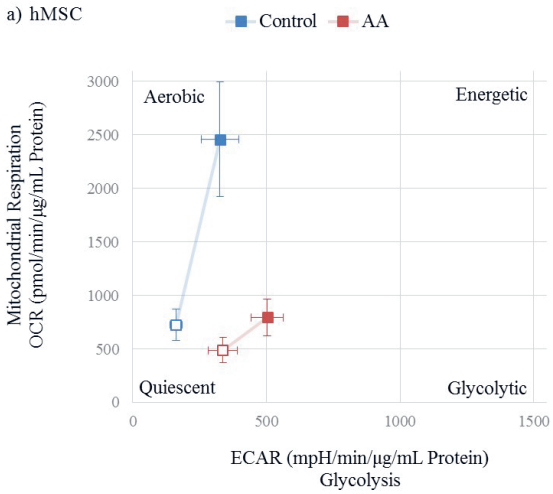

Fig. 4. Ascorbic acid effect on energy phenotype of the cells. The phenotype maps generated by Seahorse XF technology display a change in oxygen consumption rate (OCR) *i.e.*, extracellular acidification rate (ECAR) for untreated (blue) and samples treated with 10 mmol L^{-1} ascorbic acid for 24 hours (red). OCR and ECAR values are measured in baseline (open symbols) and stressed (closed symbols) conditions for: a) human mesenchymal stem cells (hMSC); b) U2OS osteosarcoma cell line; c) parental OS cells. All results are demonstrated as mean ± SD.

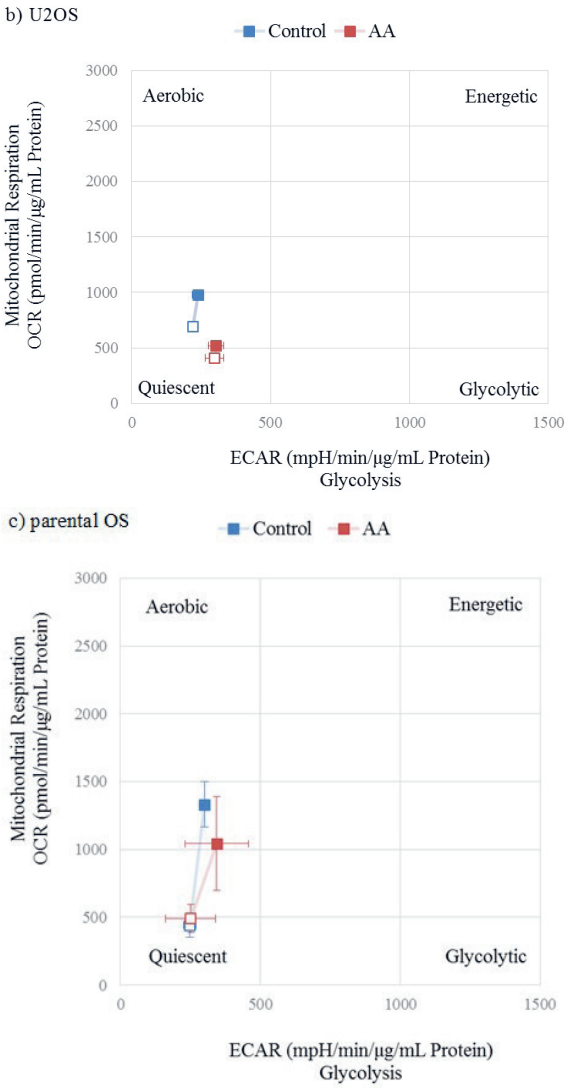

Fig. 4. Continued

24-hour incubation of OS-CSC with 10 mmol L^{-1} caused just a slight decrease in baseline values. In response to mitochondrial stressors, both ECAR and OCR values decreased in the group that received AA treatment. Since the baseline OCR/ECAR ratio is higher than 4, the stressed ECAR for control cells could include both glycolysis and mitochondrial activity.

Based on the baseline and stressed phenotype, the metabolic potential was calculated for each of the sample groups. The metabolic potential has decreased in both its glycolytic

and aerobic metabolic components in treated hMSC cells. The glycolytic potential of U2OS cells treated with AA roughly decreased, but a notable decrease in aerobic metabolic potential was observed. Treated parental OS samples exhibited almost 2 x lower aerobic metabolic potential compared to the untreated cells. A decrease in both aerobic metabolic and glycolytic potential was observed for treated OS-CSC relative to the control. OS-CSC group displayed the biggest deviations in measurements.

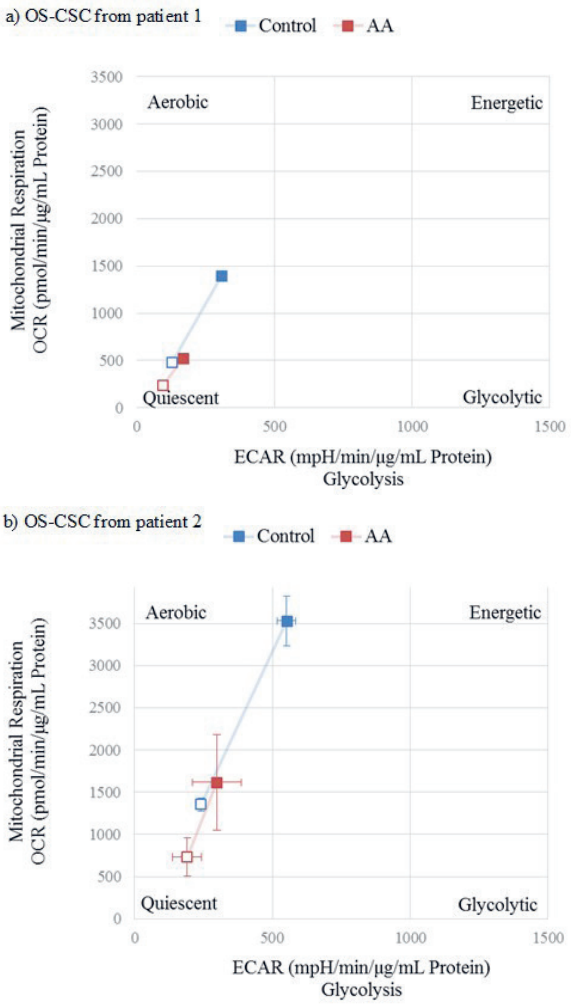

Fig. 5. Phenotype maps for osteosarcoma stem cell samples derived from three different patients. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) are measured in baseline (open symbols) and stressed (closed symbols) conditions for untreated (blue) and samples treated with 10 mmol L^{-1} ascorbic acid for 24 hours (red) using the Seahorse technology. a) Patient 1; b) patient 2; c) patient 3. All results are demonstrated as mean ± SD.

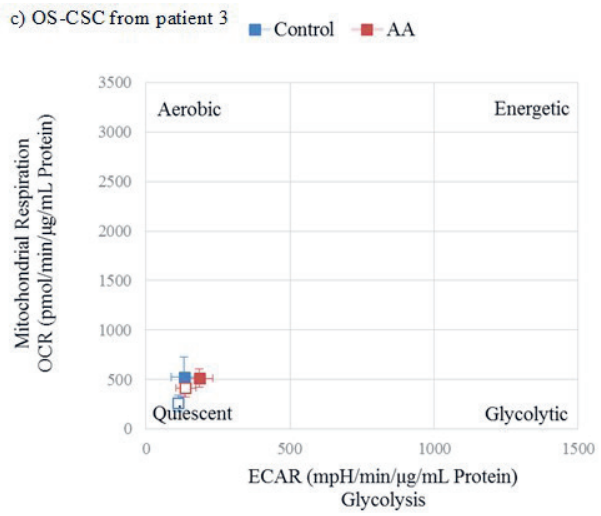

Fig. 5. Continued

Being our main group of interest, we wanted to further dissect its metabolic behavior in control and treated conditions by observing OS-CSC from each of the patients separately (Fig. 5). Energy phenotypes for OS-CSC derived from different patients differ from one another. When represented on the same scale, it is evident that patient 3 with the lowest OCR and ECAR values showed the most quiescent phenotype, while cells from patient 2 came off as the most energetic (the highest OCR and ECAR values). Patient 1 and patient 2 displayed a decrease in both glycolytic and aerobic components of metabolic potential for treated cells relative to untreated.

Using the sarcosphere culture system, OS-CSC were isolated from patients' biopsies. The stemness of these cells was confirmed by the first and the second generation growth under nonadherent conditions, and by the Hoechst 33342 dye uptake assay. Finally, after isolation and identification of CSC, testing the effect of the presumptive targeting compound can be conducted. Even though there are some indications that AA can induce terminal differentiation (15), the aim of this research was to study its cytotoxic effect. With this intention, an MTT assay was performed.

Cell type resistance to AA was following: U2OS > HEK293 > hMSC > parental cells > OS-CSC. Even though differences between IC_{50} values for different cell types are not notable, with the lowest IC_{50} value of 15.5 mmol L^{-1}, OS-CSC showed the highest sensitivity to the AA treatment among tested cell types. Fernandes *et al.* observed antiproliferative, antidifferentiation, and apoptotic effects of AA on the G292 OS cell line by using the concentration of 1 mmol L^{-1} (16). The lowest concentration used in this study was 5 mmol L^{-1}. This concentration showed almost no effect on cell viability either of the parental OS cells or OS-CSC, while at the same time percentage of live U2OS cells decreased by approximately 20 %. Likewise, Lee *et al*. showed that a higher AA concentration (≥ 10 mmol L^{-1}) was needed to decrease the viability of breast cancer cells (17). In our study, OS-CSC showed some sensitivity to AA compared to other cell types, but this difference in sensitivity was

not excessive, so it seems that AA does not act selectively on OS-CSC. According to Fenton's reaction, vitamin C can also damage cell structures by acting as an ascorbyl radical regardless of cell type, and that should be kept in mind (18).

To draw any conclusions about if and how AA affects cell metabolism, Seahorse XF Cell Energy Phenotype Test was conducted. Incubation with 10 mmol L^{-1} AA did not show a significant selectivity towards any cell type in the terms of metabolism alternation. AA decreased both glycolytic and aerobic metabolic potential of all the cell types with the exemption of a glycolytic potential in parental OS samples.

We have also observed that OS-CSC from three patients caused fairly big standard deviations when presented as one and gave rise to different phenotype maps when presented separately. Not only do the CSC contribute to the heterogeneity of the tumor bulk, but this cellular population is heterogeneous within itself. Their variability and plasticity make them resistant to eradication (19). Finally, it appeared that OS-CSC from our study mostly rely on OXPHOS to generate energy. Following biopsy rules, we obtained representative tumor biopsies for histopathology diagnostics, avoiding necrotic (hypoxic) tissue where OS-CSC are believed to be, and still found them. This finding is contrary to the results obtained by Zhong *et al.* and Mizushima *et al.* (20, 21).

The differences in the results of the studies of the metabolic profiles of OS-CSC have several explanations that should be kept in mind when conducting similar studies. One proposed explanation is the potential influence of experimental conditions. Another cause may be the lack of phenotypic homogeneity and precision in defining OS-CSC and the different techniques used to isolate them. Also, there's the influence of the stages of differentiation/dedifferentiation at the moment of the sample obtaining and culturing. The fourth factor is the microenvironment because the metabolic status of OS-CSC differs in normoxic, hypoxic, and metastatic sites, and tumor stroma plays a role too.

It is also important to consider the influence of substrates used in culture media during the experiment on OS-CSC. In this study, we added basic fibroblast growth factor (bFGF) to OS-CSC to preserve their stemness, which might have influenced OS-CSC population characteristics *in vitro* and their sensitivity to AA (22, 23).

CONCLUSIONS

Studies on therapeutic options for osteosarcoma mentioned in the literature, such as metabolism targeting using vitamin C, could be more informative if they are based on tissue samples taken directly from patients because of the tumor complexity and variability.

This study showed that osteosarcoma stem cells can express various metabolic phenotypes and that their unambiguous response to vitamin C treatment cannot be expected.

Further studies on the effects of vitamin C on osteosarcoma cells grown from patients' tumor samples are needed before it is introduced as a co-therapeutic agent *in vivo*.

Conflict of interests. – The authors declare no conflict of interests.

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Authors' contribution. – M. Š. J. and L. L. wrote the paper, I. U. designed the research, M. A., M. P. and M. L. did all the experiments, L. L did data analyses, V. T. helped with the article corrections, M. Š. J., T. R. and R. K. obtained tissue samples, M. Š. J. prepared informed consents and obtained permission from the ethics committee.

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