

# Učinci askorbinske kiseline na matične stanice osteosarkoma

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**University of Zagreb**  
**Faculty of Science**  
**Department of Biology**

Karlo Skube

**The effects of ascorbic acid on osteosarcoma stem cells.**

**Učinci askorbinske kiseline na matične stanice osteosarkoma.**

Graduation thesis

Zagreb, 2018

This research is performed at Division of Molecular Biology of Faculty of Science, University of Zagreb, under the supervision of Inga Marijanović, PhD, Assist. Prof. Thesis is submitted on evaluation to Department of Biology of Faculty of Science, University of Zagreb in order to acquire the academic title of Master of molecular biology.

*I would like to thank my mentors. Inga, Maja, Kate and Majica. Thank you for guiding me through the last year. You were my second family. I can only hope to work with people like you one day.*

*I particularly want to thank my parents, Branka and Zoran, my sister Lea and my grandma Tajica. Your unconditional love and support throughout my life helped me learn true values in life. You made me a better person and without you, none of this would be possible. I love you.*

*I thank my friends. Ana, Helena, Margi and Martina. I've known you for 17 years and you've always been here. Thanks for sticking around. To my molecool girls, Adri, Ana, Anja, Lea, Luce and Viki. We made it. We survived. To my Spanish crew, Dora, Juran and Lucija for having the best semester of our lives.*

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# TEMELJNA DOKUMENTACIJSKA KARTICA

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Sveučilište u Zagrebu

Prirodoslovno - matematički fakultet

Biološki odsjek

Diplomski rad

## UČINCI ASKORBINSKE KISELINE NA MATIČNE STANICE OSTEOSARKOMA

Karlo Skube

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Matične stanice raka su stanice raka koje imaju karakteristike zdravih matičnih stanica u ljudskom organizmu. Mogućnost samoobnavljanja i diferencijacije u različite tipove stanica osiguravaju im tumorigenost, dok niska stopa proliferacije uz aktivne membranske pumpe i enzime popravka osigurava otpornost na konvencionalne kemoterapeutike. Njihova prisutnost je dokazana u osteosarkomima te se mogu izdvojiti iz tumorske mase na osnovi sposobnosti rasta u sferama - sarkosferama. S obzirom da se radi o izuzetno malignim tumorima, razvoj specifičnih terapija koje ciljaju tu populaciju stanica neophodan je za poboljšanje preživljenja pacijenata. S obzirom na njihove mehanizme otpornosti teško ih je selektivno ciljati. Ovim istraživanjem ispitan je utjecaj askorbinske kiseline na matične stanice osteosarkoma te njezina selektivnost u ovisnosti o koncentraciji askorbinske kiseline te broju i porijeklu stanica. Djelovanje askorbinske kiseline utvrdili smo testovima vijabilnosti, esejom inhibicije rasta sarkosfera, protočnim citometrom, western blotom i mjerenjem razine reaktivnih kisikovih vrsta. Zaključno, askorbinska kiselina uzrokuje apoptozu te je njena letalna koncentracija ovisna o broju matičnih stanica osteosarkoma. Također, razina GAPDH proteina je nedetektibilna što ukazuje na inhibiciju glikolize. Razina reaktivnih kisikovih vrsta povišena je kod stanica tretiranih askorbinskom kiselinom te se može zaključiti da djeluje kao oksidans.

(32 stranice, 11 slika, 38 literaturnih navoda, jezik izvornika: engleski)

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**Ključne riječi:** sarkosfere, vijabilnost, GAPDH, tumor, glikoliza

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**Neposredni voditelj:** dr. sc. Maja Antunović

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## BASIC DOCUMENTATION CARD

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University of Zagreb

Faculty of Science

Division of Biology

Graduation thesis

### THE EFFECTS OF ASCORBIC ACID ON OSTEOSARCOMA STEM CELLS

Karlo Skube

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Cancer stem cells are cancer cells that have the characteristics of healthy stem cells in the human body. The possibility of self-replication and differentiation in different cell types ensures their tumorigenicity while low proliferation rates with active membrane pumps and repair enzymes provide resistance to conventional chemotherapy. Their presence is proven in osteosarcoma and can be extracted from tumor mass based on the ability to grow in spheres - sarcospheres. Given that it is an extremely malignant tumor, the development of specific therapies targeting that population of cells is necessary to improve patient survival. Due to their resistance mechanisms, it is difficult to selectively target them. This study investigated the effect of ascorbic acid on osteosarcoma stem cells and its selectivity depending on the concentration of ascorbic acid and the number and origin of cells. The effects of ascorbic acid have been determined by viability tests, sarcosphere growth inhibition assays, flow cytometry, western blot and measurement of reactive oxygen species. In conclusion, ascorbic acid causes apoptosis and its lethal concentration is dependent on the number of osteosarcoma stem cells. Also, the level of GAPDH protein is undetectable, which suggests that ascorbic acid inhibits the pathway of glycolysis. The level of reactive oxygen species is increased in cells treated with ascorbic acid and it can be concluded that it acts as an oxidant.

(32 pages, 11 figures, 38 references, original in English)

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**Keywords:** sarcospheres, viability, GAPDH, tumor, glycolysis

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## Table of Contents

<b>1. INTRODUCTION .....</b>	<b>1</b>
<b>1.1. Osteosarcoma .....</b>	<b>1</b>
1.1.1. Etiology .....	1
1.1.2. Treatment .....	2
<b>1.2. Cancer Stem Cells .....</b>	<b>2</b>
1.2.1. Stochastic versus CSC theory .....	3
1.2.2. Therapeutic targets .....	4
1.2.3. Metabolism as a target .....	5
<b>1.3. Ascorbic acid and cancer .....</b>	<b>6</b>
<b>2. OBJECTIVE OF THE STUDY .....</b>	<b>8</b>
<b>3. MATERIALS AND METHODS .....</b>	<b>9</b>
<b>3.1. Sarcosphere formation assay .....</b>	<b>9</b>
<b>3.2. Sarcosphere isolation and adhesion .....</b>	<b>9</b>
<b>3.3. MTT viability assay .....</b>	<b>9</b>
<b>3.4. Sarcosphere inhibition assay .....</b>	<b>10</b>
<b>3.5. Western blot analysis of GAPDH expression.....</b>	<b>10</b>
3.5.1. Cell seeding and treatment .....	10
3.5.2. Cell lysis and protein isolation .....	11
3.5.3. Bicinchoninic acid (BCA) assay for determination of protein concentration.....	11
3.5.4. Protein separation by polyacrylamide gel electrophoresis (PAGE) and protein transfer .....	11
3.5.5. Antibody incubation and detection .....	12
3.5.6. Membrane stripping .....	13
<b>3.6. Detection of reactive oxygen species by DCFH-DA assay .....</b>	<b>13</b>
<b>3.7. Determination of cell death type by Annexin V and PI assay .....</b>	<b>14</b>
<b>4. RESULTS .....</b>	<b>15</b>
<b>4.1. Sarcosphere formation and adhesion .....</b>	<b>15</b>
<b>4.2. Ascorbic acid effect on cell viability.....</b>	<b>17</b>
<b>4.3. Sarcosphere growth inhibition in the presence of AA .....</b>	<b>20</b>
<b>4.4. Effects of AA on glycolytic cycle .....</b>	<b>21</b>
<b>4.5. Determination of cell death type .....</b>	<b>22</b>
<b>4.6. The effects of AA on redox state of OS-CSCs.....</b>	<b>24</b>
<b>5. DISCUSSION.....</b>	<b>25</b>

**6. CONCLUSION .....28**  
**7. REFERENCES.....29**



# 1. INTRODUCTION

## 1.1. Osteosarcoma

Osteosarcoma is a malignant tumor of mesenchymal origin. Immature osteoid, which is the non-mineralized, organic portion of the bone matrix, is produced by the tumor cells. The occurrence of osteosarcoma is bimodal. It is being found predominantly in children and young adults and in elderly individuals which makes it the most common primary sarcoma. Depending on the degree of aggressiveness, there are several different types of osteosarcoma. Most of the patients, 80% of them, have micro-metastatic or metastatic disease at diagnosis which makes the treatment strategies very similar among them. Therapies are consisting of multiagent chemotherapy in addition to surgical removal of the affected area. The number of amputations has decreased significantly with the advances in surgical treatment. Since the description of osteosarcoma in 1800's until today, the prognosis for patients has improved from less than 30% survival rate to more than 70%. Considering the anatomic location of osteosarcoma, it seems it has a preference for the metaphyseal part of the long bones (Peabody & Attar, 2014).

### 1.1.1. Etiology

The problem with osteosarcoma is that it's true etiology is unknown. It is considered to be multifactorial and while some sarcomas have demonstrated consistent chromosomal translocations or abnormalities, the majority of osteosarcomas are aneuploid (Kansara & Thomas, 2007). Ottaviani & Jaffe (2002) did a clinical and pathologic study of brother and sister with osteosarcoma and concluded that there seems to be a molecular basis for the development of osteosarcoma. For example, Hansen et al. showed that *RB* gene who regulates the transition between G<sub>0</sub>/G<sub>1</sub> and S phase of the cell cycle increased the risk of osteosarcoma for 500 times. Wadayama et al. (1994) detected mutations in *RBI* gene in sporadic cases of osteosarcoma. Other mutations that can predispose a person to develop osteosarcoma include inactivation of the p53 tumor suppressor, MDM2, p14<sup>ART</sup> and CDK4 (Kansara & Thomas, 2007). Wang reported that DNA helicase abnormalities caused by mutations in *RECQL2*, *RECQL3*, and *RECQL4* have also been associated with osteosarcoma. On the other hand, osteosarcoma can be environmentally caused and the most common cause is ionizing radiation (Peabody & Attar, 2014).

### **1.1.2. Treatment**

Before the 1970s the outcome for patients with osteosarcoma was not so bright. Only about 20% of affected people had a 5-year survival rate. With the discovery of methotrexate (MTX) survival rates have improved significantly, up to 65%. MTX is antifolate which means it functions as a folic acid antagonist. It suppresses dihydrofolate reductase (DHFR) whose role is to catalyze tetrahydrofolate synthesis and therefore inhibits the synthesis of RNA, DNA and proteins (Friedman & Carter, 1972; Goorin et al., 1987; Sutow, 1980). Today, there is no singular regimen for osteosarcoma. Agents that are most commonly used include MTX, doxorubicin, cisplatin and ifosfamide. Also, other agents include vincristine, bleomycin and cyclophosphamide (Maki, 2012).

Effectiveness of the chemotherapy is highly dependent on the surgical restriction of the tumor as well as the reconstruction of the resulting anatomical defect. Wide excisions are becoming more and more popular because they keep the local disease controlled in 95% of the time and decrease recurrence possibility. They are used when there is no threat to neurovascular structures (Bacci et al., 2006).

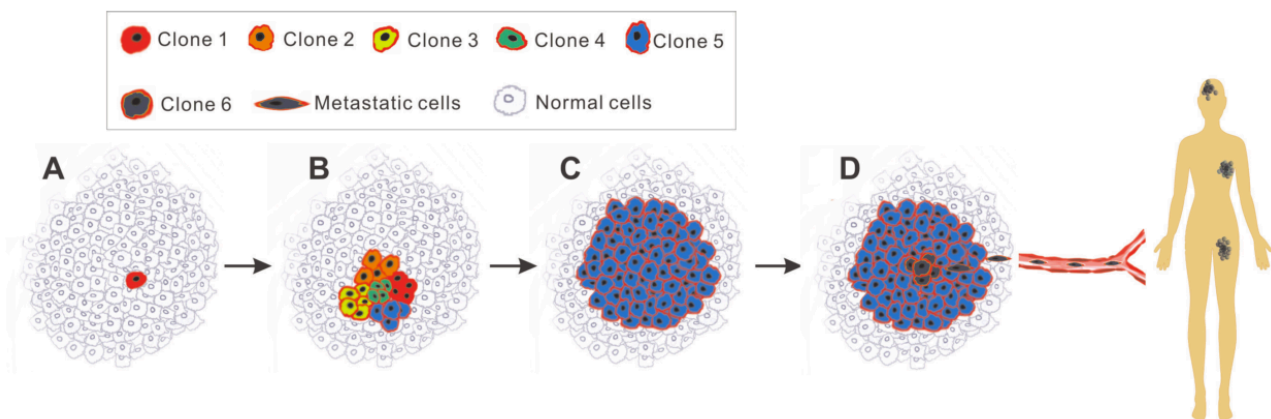
## **1.2. Cancer Stem Cells**

Stem cells, cancer or not, have two main properties. One of them being the ability to self-renew and the second one, the ability to give rise to progenitors of differentiated cell types of one or more lineages. The self-renewal feature rises two proposals that are important to stem cell carcinogenesis. The first one states that the self-renewal property increases the opportunities for cancer to accumulate more carcinogenic changes, while the second one suggests that altered self-renewal regulation directly underlies carcinogenesis. Being able to self-renew gives cells enough time to accumulate mutations and pass them to daughter cells. Losing the balance between self-renewal and differentiation can lead to unregulated self-renewal which is a hallmark of cancer. On the other hand, Takahashi et al. (2007) showed that differentiated cells can undergo the mutation that reactivates a self-renewing program and in that way cause cancer. These proposals are supported by the identification of cancer stem cells - a very small population of cells within a tumor with the ability to self-renew and give rise to all cell types found in a tumor and therefore cause tumor recurrence. That ability makes them tumorigenic or tumor-forming, in contrast to other non-tumorigenic cancer cells. Metabolism in CSCs is altered as well and can be used as a therapeutic target. CSCs switch mainly to aerobic

glycolysis (Pecorino, 2012). Markers specific for healthy stem cells are used for identification of CSCs. Markers that showed good results in isolating CSC-enriched populations are CD133, CD44, CD24, THY1, ABCB5 and CD200 (Al-Hajj et al., 2003). CSCs can be identified by other methods as well. Fluorescence-activated cell sorting (FACS) is used to identify CSCs by fluorescently bound antibodies that are directed on cell markers. Hoechst dye is removed from CSCs by ATP-binding cassette (ABC) transporters and is used as well for identification of CSCs. Sphere formation is the ability of CSCs to grow in non-adherent conditions and these assays are used to isolate CSCs from other cancer cells (Nicolis, 2007).

### 1.2.1. Stochastic versus CSC theory

Anti-cancer therapy has been directed by stochastic, or clonal evolution, theory for decades (Fig. 1). It states that at least five mutations are needed to occur in healthy somatic cells for them to gain cancer phenotype such as enhanced proliferation, reduced capacity to undergo apoptosis and inhibition of differentiation (Hanahan, 2011). The drawback of this theory is that somatic cells have limited lifespan and it would be almost impossible for them to acquire all

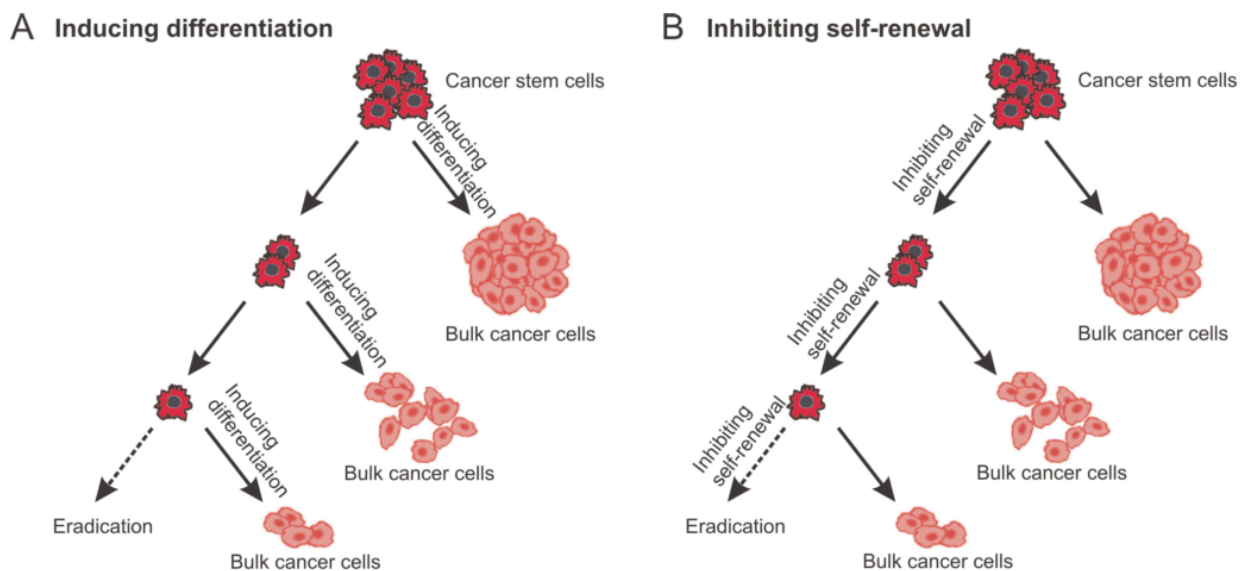


**Figure 1.** Stochastic theory of cancer development states that each cell in tumor has similar tumorigenic capacity. **A** - transformation of normal cell into tumor cell (red). **B** - cancer cell proliferates and produces clones, but because of genetic instability produces various other clones. **C** - a selective pressure such as hypoxia or chemotherapy eliminates badly adapted clones. **D** - additional selection gives rise to new cancer cells (Clone 6) with aggressive phenotype and ability to metastasize. Adapted from Wang et al., 2015.

the necessary mutations for them to become cancer cells. The explanation of this could be that the first mutation that hits the cell is the one that gives the cell the ability to be immortal (Tomasetti et al., 2013). Following this leads to the conclusion that every viable tumor cell has the same capability of forming a new tumor. This, on the other hand, contradicts the well-known fact that it usually takes more than 10 000 cancer cells to initiate tumor in immunocompromised mice. This led to new ideas proposed by the CSC theory (Wang et al., 2015).

### 1.2.2. Therapeutic targets

Therapies that target CSC are based on the broad knowledge and understanding of tumor genetics and signaling pathways and are generally classified according to the therapeutic strategies. The first one is the so-called “destemming” of CSCs (Fig. 2). It includes two aspects, one being the promotion of CSC differentiation into non-CSCs while the other one targets inhibition of their self-renewal properties. In CSCs, self-renewal pathways are similar to those in normal stem cells, some of them have been studied very thoroughly. By far the most known ones associated with CSC are Notch, Hedgehog and Wnt/ $\beta$ -catenin signaling pathways. Vismodegib is a Hedgehog inhibitor that has been approved in the clinic for the treatment of basal cell carcinoma (Dubey et al., 2013). Some Notch inhibitors such as  $\gamma$ -secretase inhibitors and monoclonal antibodies have been evaluated in the past few years. Monoclonal antibody



**Figure 2.** “Destemming” cancer stem cells. Two therapeutic approaches include induction of differentiation (A) and inhibition of self-renewal (B). Adapted from Wang et al., 2015.

Tarextumab in combination with conventional chemotherapeutics showed stabilization or shrinkage of the pancreatic tumor over the period of as long as 12 months in 83% of 29 patients (Yen et al., 2015). As for the second approach, the differentiation of CSCs, the most studied signaling pathways are bone morphogenic protein (BMP) and oncostatin M (OSM) pathways. Lombardo et al. (2011) observed an increase in terminal differentiation through the simulation of BMP signaling by BMP4 in colorectal CSCs as well as the better chemotherapeutic sensitivity (Wang et al., 2015).

Accumulation of genomic and epigenetic mutations enhances drug resistance of CSCs. Inhibition of self-renewal and promotion of differentiation can increase chemosensitivity of CSCs but there are other approaches that target drug resistance mechanism directly. ABC transporters are known to be able to pump out exogenous small molecules such as chemotherapeutics. ABCB2 is known to cause glioblastoma CSC resistance to a variety of chemotherapeutics. ABCB5 is used as a CSC marker for melanoma CSCs (Schatton et al., 2008). Unfortunately, not a lot of success has been made in targeting ABC transporters and rare clinical benefit has been recorded. On the other hand, deregulation of intrinsic and extrinsic apoptosis pathways gives promising results. Most types of CSCs show overexpression of the Bcl-2 family of proteins which are a group of anti-apoptotic proteins related to the critical step of the intrinsic apoptotic pathway (Zeuner et al., 2014). ABT-199, ABT-737 and TW-37 are Bcl-2 inhibitors and have shown great targeting capacity. For example, ABT-737 alone was able to reduce the content of CSCs in treated acute myeloid leukemia (AML) as well as in lung and breast tumors (Li et al., 2012). Inhibitor of apoptosis protein (IAP) is the last line of defense against apoptosis as it prevents both intrinsic and extrinsic apoptosis by inhibition of caspases (Berthelet & Dubrez, 2013). The ones drawing attention the most are XIAP and survivin, homologs of IAP family. Interest thing about survivin is that it overexpresses specifically in human cancers and does not in most adult tissues. That property makes it an attractive target for anticancer therapies. Secondly, survivin has been proposed to be an important stem cell-associated gene and in combination with Hiwi and hTERT genes, the risk of death in patients with soft-tissue sarcoma has been significantly increased (Taubert et al., 2010).

### **1.2.3. Metabolism as a target**

Warburg effect describes a metabolic alteration in cancer cells. Healthy cells normally produce energy through mitochondrial oxidative phosphorylation. However, cancer cells carry out aerobic glycolysis, where they convert glucose to lactate even in the presence of oxygen.

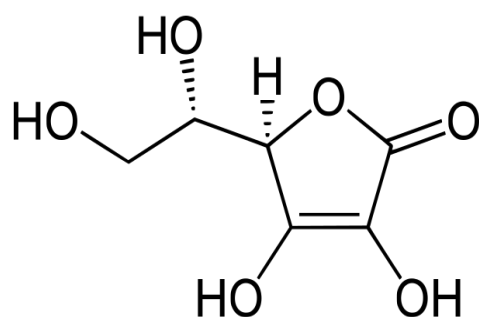
The Warburg effect has been studied, but its true nature still remains unknown. Reprogramming energy metabolism is one of the hallmarks of cancer and 60-90% of tumors switch to glycolysis. It is proposed that the metabolism of cancer cells resembles the metabolism of proliferating cells. Some think that the shift is time-dependent, while the others suggest it may be cell-type-dependent as they both cancer cells and proliferating cells need to adapt to the anabolic need for proliferation. Knowing this, glycolysis is a popular target for blocking the main energy metabolism pathway in tumor cells. There is evidence that response to hypoxia via AMP-activated protein kinase (AMPK) contributes to altered metabolism. AMPK senses the increased levels of AMP and decreased levels of ATP which is a sign of starvation and hypoxia. AMP binds to the  $\gamma$  regulatory subunit of AMPK and uncovers the phosphorylation site on the  $\alpha$  subunit of AMPK. LKB1 phosphorylates the  $\alpha$  subunit and thus activates AMPK. AMPK then acts as a transcriptional factor and inhibits ATP-consuming pathways and activates ATP-generating pathways such as glucose (Pecorino, 2015).

### **1.3. Ascorbic acid and cancer**

Ascorbic acid (AA), L-ascorbic acid or vitamin C is a vitamin found in food and used as a dietary supplement (Fig 3.). It is absorbed in the body by active transport and simple diffusion. Hexose transporters GLUT1, GLUT3 and GLUT4 are used for import of dehydroascorbic acid (DHA) and then it is reduced to ascorbic acid by different enzymatic systems. Sodium-dependent vitamin C transporters (SVCT1 and SVCT2) are used to transport ascorbate and are used more than GLUT transporters (Savini et al., 2008). Bonuccelli et al. (2015) showed that AA acts as a glycolysis inhibitor in CSC. It induces oxidative stress which inhibits the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme.

Ascorbic acid has been widely used as an antitumor agent but comprehensive clinical results are still not existing. Mata et al. (2015) did a systematic review of clinical and non-clinical studies about the role of AA in the prevention and/or treatment of cancer between 2011 and 2015. The study was split into two categories. The first one summed up studies that described AA as an antioxidant, preventing oxidation which induces apoptosis. Clinical studies (2) involved different types of cancers and doses of AA from 0.04-10 mM. Non-clinical studies (10) involved different types of cell lines, one of them being osteosarcoma 143-B cell line. AA doses ranged from 0.05-4 mM. Mentioned mechanism of action includes decreased levels of C-reactive protein and proinflammatory cytokines, obstruction of metastases and tumor growth,

protection against lung injury induced by smoking by inhibiting the expression of cyclin D1, decreased the production of reactive oxygen species and limitation of the invasive potential. The second category consists of studies that describe AA as pro-oxidant. High concentrations of AA may increase the production of adenosine triphosphate (ATP) inducing apoptosis. Only one of these is a clinical study on patients with metastatic pancreatic cancer. They were treated with 0.28, 0.34 and 0.56 mM doses of AA and decrease in tumor size has been reported. Regarding the non-clinical studies (17), different cell lines were used and AA concentrations ranging from 5  $\mu$ M to 28.39 mM. Low, micromolar, concentration of AA was able to kill cell lines *in vitro*, while *in vivo* it generated hydrogen peroxide, superoxide radicals and extracellular ascorbyl that are responsible for its cytotoxic activity. Interestingly, concentrations as high as 20 mM did not harm non-malignant cells. Regarding the mechanisms of action, increased levels of H<sub>2</sub>O<sub>2</sub> were reported, as well as interference with cell cycle (G<sub>0</sub>/G<sub>1</sub>), decreased cell growth, induction of apoptosis, aponecrosis and autophagy and increased expression of p53.



**Figure 3.** Atomic structure of ascorbic acid

To summarize, AA can act as both reducing and oxidizing agent, depending on the concentration and the environment in which the vitamin is present. At lower concentrations, ascorbic acid displays antioxidant properties, while at higher concentrations it induces apoptosis in malignant cell lines via its pro-oxidant action (González et al., 2010). The specific mechanism still remains unclear. This study will examine the effects of ascorbic acid on OS-CSCs and try to propose a possible mechanism.

## 2. OBJECTIVE OF THE STUDY

The primary goal of this study is to find a way to eliminate osteosarcoma cancer stem cells (OS-CSCs). Their ability to self-renew and differentiate into different types of malignant cells ensures their tumorigenicity while the low proliferative rate, active pumps and DNA repair enzymes makes them resistant to conventional chemotherapeutics. There is controversial data considering the effects of ascorbic acid on prevention and treatment of tumors and based on differences in metabolic pathways between healthy cells and tumor stem cells we hypothesize that the effect of ascorbic acid could be lethal to tumor stem cells.

The specific goals of this research are:

- To expand isolated osteosarcoma stem cells from patient's tumor *in vitro* as adherent culture and as spheroids
- To test the effect of ascorbic acid on viability of osteosarcoma stem cells and determine the  $IC_{50}$  value by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay
- To test if osteosarcoma stem cells grown in low attachment conditions are susceptible to ascorbic acid and if they will be able to form spheroids
- To test which type of cell death is triggered by ascorbic acid by flow cytometry
- To analyze the mechanism of cell death induction
- To test if ascorbic acid changes the redox state of the OS-CSCs



### **3. MATERIALS AND METHODS**

#### **3.1. Sarcosphere formation assay**

Cells used were previously isolated from patient's tumor and grown in growth medium (GM, 10% FBS, 1% Pen/Strep in F12/HAM). The medium is removed by aspiration. Osteosarcoma stem cells were washed with phosphate buffer saline, (PBS, Gibco). 3 mL of trypsin-EDTA (Sigma) was added to each plate. A plate was shaken gently and trypsin was immediately removed by aspiration. The process was repeated. Cells were incubated for 4 minutes at 37 °C, 5% CO<sub>2</sub> (Panasonic). Trypsin reaction was stopped by adding 10 mL of growth medium (GM, 10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (Lonza) in F12/HAM (Biological Industries)). Cells were counted using the hemocytometer. Methyl-cellulose (MC, 2% in miliQ-H<sub>2</sub>O, Sigma) was prepared 3 days in advance and stirred for 2 days at 4 °C and autoclaved. Sarcosphere growth medium was prepared (SGM, 20% knockout serum replacement Gibco, 1% Pen/Strep, 0.01% EGF (Gibco), 0.01% bFGF (Gibco) in F12/HAM). MC and SGM were combined in the equal ratio to the final concentration of 1% MC. 40 000 cells/well were seeded in 6-well ultra-low attachment plates in 5 mL of MC-SGM. Every three days growth factors were added to the final concentration of 10 ng/mL. Sarcosphere formation was recorded by photography in the period of 31 days.

#### **3.2. Sarcosphere isolation and adhesion**

After 31 days of sarcosphere growth, they were isolated and seeded under adherent conditions to form a monolayer. GM was prepared. Sarcospheres were transferred into 40 μm nylon cell strainers (Thermo Fisher Scientific, USA) and MC-SGM was left to drip off using only gravitational force. Wells were washed 2 times with 5 mL of GM which was transferred to cell strainers as well. Cell strainer was washed with extra 10 mL of GM to wash away all single cells that remained. Cell strainer was transferred to a petri dish, rotated for 180°, washed with 10 mL of GM and gently shaken to release the sarcospheres into the petri dish. Cell strainer was observed under the microscope to make sure all the sarcospheres were transferred. Cells were incubated at 37 °C and 5% CO<sub>2</sub>.

#### **3.3. MTT viability assay**

Cells were seeded on the 96-well plate at the cell density of  $0.25 \times 10^4$  cells/well in stem cell growth medium (SCGM, 10% FBS 1% Pen/Strep, 0.01% bFGF). After 24 hours cells were

treated with ascorbic acid (AA) in a concentration ranging from 2.5 µg/mL to 55 µg/mL in quadruplicates. Control cells had their medium refreshed without the addition of AA. Cells were incubated for 72 hours at 37 °C and 5% CO<sub>2</sub>. After incubation, the medium was removed by aspiration and 40 µL of 1x MTT (Sigma) was added to each well and cells were incubated for 3.5 h at 37 °C and 5% CO<sub>2</sub>. After MTT incubation, 170 µL of dimethyl sulfoxide (DMSO, Roth) was added to each well and incubated at 37 °C for 30 minutes. After the dissolution of formazan in DMSO, the intensity of purple color was measured on GloMax microplate reader (Promega), λ = 560 nm. Inhibition concentration where 50% of the cells are dead (IC<sub>50</sub>) was calculated. The same procedure was done with HEK293, hMSC and U2OS cell lines. To test the effects of AA depending on cell density, the experiment was carried out one more time with seeding densities of 0.25 x 10<sup>4</sup>, 0.5 x 10<sup>4</sup> and 1 x 10<sup>4</sup> cells/well. Concentrations of AA were the same and only osteosarcoma stem cells were used.

### **3.4. Sarcosphere inhibition assay**

One 6-well ultra-low attachment plate was seeded with osteosarcoma stem cells as described in paragraph 3.1. MTT assay results were used to calculate the amount of AA that was absorbed by each cell at AA concentration of 30 µg/mL and 40 µg/mL which was 0.0048 µg/cell and 0.192 µg/cell, respectively. Each treatment was done in duplicates and two control wells were untreated. Cells were incubated at 37 °C and 5% CO<sub>2</sub>. Every three days, the concentrations of AA were replenished and sarcosphere growth progression was photographed.

### **3.5. Western blot analysis of GAPDH expression**

#### **3.5.1. Cell seeding and treatment**

Cells were seeded on the 6-well plate at the cell density of 2 x 10<sup>5</sup> cells/well. Cells in each well had its own treatment. First, two wells were treated with 30 µg/mL and 40 µg/mL, respectively. Next two wells were treated with the same concentration of AA as the first two with added 10 µM of cisplatin (Sigma). The fifth well was treated only with 10 µM cisplatin while the last one was untreated and used as a control. Cells were incubated at 37 °C and 5% CO<sub>2</sub>.

### **3.5.2. Cell lysis and protein isolation**

After 72 hours of incubation, cells were lysed using CellLytic™ M (Sigma, USA) reagent. The medium from each well was transferred to a different tube, washed with 2 mL of PBS and transferred as well. 1 mL of trypsin-EDTA solution was added to each well and incubated for 4 min. Trypsin was neutralized with 1 mL of medium and transferred to tubes. Wells were once again washed with 2 mL of PBS and transferred to tubes. Tubes were centrifuged for 10 min at 450 x g (Universal 320R, Hettich) at 4 °C. The supernatant was aspirated. In a separate tube, 400 µL of CellLytic™ M reagent was mixed with 4 µL of Protease Inhibitor Cocktail. 30 µL of CellLytic™ M and Protease Inhibitor Cocktail mix was added to each pellet, resuspended well, transferred to clean tubes and frozen at -20 °C. Proteins were isolated using the freeze-thaw method. Cell lysates are defrosted on the ice, transferred to liquid nitrogen and defrosted again. The process was repeated three times vortexing the samples each time before freezing. Tubes were centrifuged 15 min at 20 000 x g at 4 °C. The supernatant was transferred to clean tubes.

### **3.5.3. Bicinchoninic acid (BCA) assay for determination of protein concentration**

BCA assay (Santa Cruz) was used to determine protein concentration in each sample. Working reagent (WR) volume was calculated based on the number of standards (9) and samples (six samples times two replicas). Reagent A and B were mixed in 50:1 ratio and kept in the dark. Standards and samples are diluted 5 times in miliQ water. 10 µL of diluted samples and 200 µL of WR is added to each well in 96-well plate. Samples are incubated at 37 °C for 30 min. Color intensity was measured on GloMax microplate reader,  $\lambda = 560$  nm and protein concentration was calculated.

### **3.5.4. Protein separation by polyacrylamide gel electrophoresis (PAGE) and protein transfer**

The volume needed for 5 µg of protein was calculated from the BCA assay. Stock volume was mixed with the equal amount of protease inhibitor and lysis buffer cocktail. Double the volume of protein stock of sample buffer was added to the mixture. Total dilution is 4 times. Samples are incubated at 95 °C for 5 min in thermoblock (Eppendorf). Samples were chilled on ice and spun down. Total volume was transferred to the wells. The gel used was Amersham

ECL Gel 4-20% with 10 wells (GE Healthcare Life Sciences). 1x running buffer was prepared by diluting 19 mL of 10x Amersham ECL Gel Running Buffer (GE Healthcare Life Sciences) in 171 mL of water. 90 mL of 1x running buffer was added to each tank of Amersham ECL Gel Box (GE Healthcare Life Sciences). Gel cassette was rinsed with distilled water and placed in gel box so that the well side of the cassette faces toward the cathode. Gel was pre-run for 12 min at 160 V. After the pre-run, comb was removed and 6 mL of 1x running buffer was added to the well container. Samples were loaded onto the gel. Gel has been running for 60 min at 160 V at 4 °C. Once the run completed the stacking gel and the front were cut off. The gel is gently laid down on a pre-soaked filter paper in transfer buffer (3.35 g TRIS, 14.4 g Glycine, 100 mL methanol in 1 L of deH<sub>2</sub>O) and covered with transfer buffer to keep its moisture. Pre-soaked nitrocellulose membrane (GE Healthcare Life Sciences) in transfer buffer is placed on top of the gel. Air bubbles were removed by gently rolling a glass pipette over the membrane surface. Another pre-soaked filter paper is placed on top of the membrane. Four soaked blotting pads are placed into the cathode core of the XCell II™ Blot Module (Invitrogen). The gel-membrane “sandwich” is carefully placed on top of the blotting pads, such that the gel is closest to the cathode plate. Four blotting pads are added on top of the “sandwich.” Anode core is placed on top of the pads and the whole blot module is placed into the guide rails on the lower buffer chamber. Blot module is filled with transfer buffer until the sandwich is covered. The outer, buffer, chamber is filled with distilled water to the designated mark. The lid is placed on top of the unit and the whole unit is placed in styrofoam box filled with ice. The transfer was done at 10 V overnight.

### **3.5.5. Antibody incubation and detection**

After the transfer has completed, blot module was disassembled and the membrane was incubated with Ponceau S (Sigma) dye for 5 min to visualize the proteins. The membrane is destained by washing in distilled water 3 times for 3 min and once in PBS. Blocking was done in 5% non-fat dry milk dissolved in TBST for 1 hour. The membrane was washed in TBST two times for 5 min. Primary GAPDH antibody (Sigma) was diluted in 0.5% non-fat milk in TBST in ratio 1:10000. The membrane was covered with primary antibody aliquot and incubated overnight at 4 °C. The membrane was washed 3 times with TBST for 5 min. 5 mL of 5% non-fat dry milk was prepared. Milk was added to the membrane and 2.5 µL (1:2000 dilution) of secondary anti-rabbit IgG, HRP-linked antibody (Cell Signaling, prod. #7074) was added to milk TBST solution. The membrane was incubated for 1 hour at room temperature. The

membrane was washed 3 times with TBST for 5 min. Clarity Western Peroxide Reagent and Clarity Western Luminol/Enhancer Reagent (Biorad) were mixed in the equal ratios and kept in dark at room temperature. The membrane was dried on a paper towel after washing and placed on plastic wrapping foil. The membrane was covered with Clarity mixture, covered with another layer of plastic foil and incubated for 3 minutes in the cassette. Excess Clarity mixture was removed but the membrane remained wet, wrapped in clean plastic foil and placed in the cassette. It needs to be sure that there are no air bubbles trapped between the membrane and the foil. X-ray film (Kodak) was placed on top of the membrane under the red light and exposed for 20 min. In the meantime, 22 mL of the developer (Sigma) was mixed with 100 mL of water. The same was done with the fixer (Sigma). Both were placed in separated plastic containers while the third one contained only water. After exposure, X-ray film is washed in the developer until the bends are starting to appear. The film is then quickly washed in the water and the development reaction is stopped in the fixer. The whole procedure was repeated with actin H-196 primary antibody (Santa Cruz) diluted 1:500 in 0.5% non-fat milk in TBST after stripping the membrane as described in paragraph 3.5.6.

### **3.5.6. Membrane stripping**

Stripping is done to make sure there are no leftover primary antibodies bound to the membrane. The membrane is incubated in mild stripping buffer (15 g glycine, 1 g SDS, 10 mL Tween 20 in 1 L of distilled water, pH 2.2) two times for 10 min. Following, the membrane is incubated two times for 10 min in PBS after which is incubated two times for 5 min in TBST. When the stripping process is done, the membrane is ready for blocking stage as described in paragraph 3.5.5.

## **3.6. Detection of reactive oxygen species by DCFH-DA assay**

Intracellular ROS generation was determined by a fluorometric microplate assay by assessing oxidation of 2', 7'-dichlorofluorescein-diacetate (DCFH-DA) (Sigma-Aldrich). In brief, cells were incubated with 200  $\mu$ M DCFH-DA in PBS at 37°C for 30 min in the dark and then washed with PBS to remove excess dye. Fluorescence was measured using Glomax microplate reader (Promega). Cells were seeded on the 96-well opaque plate at the cell density of  $0.25 \times 10^4$  cells/well. Cells were treated with 30  $\mu$ g/mL and 40  $\mu$ g/mL concentrations of AA. Time of incubation was 24 h. Only propidium iodide (PI) negative cells were considered.

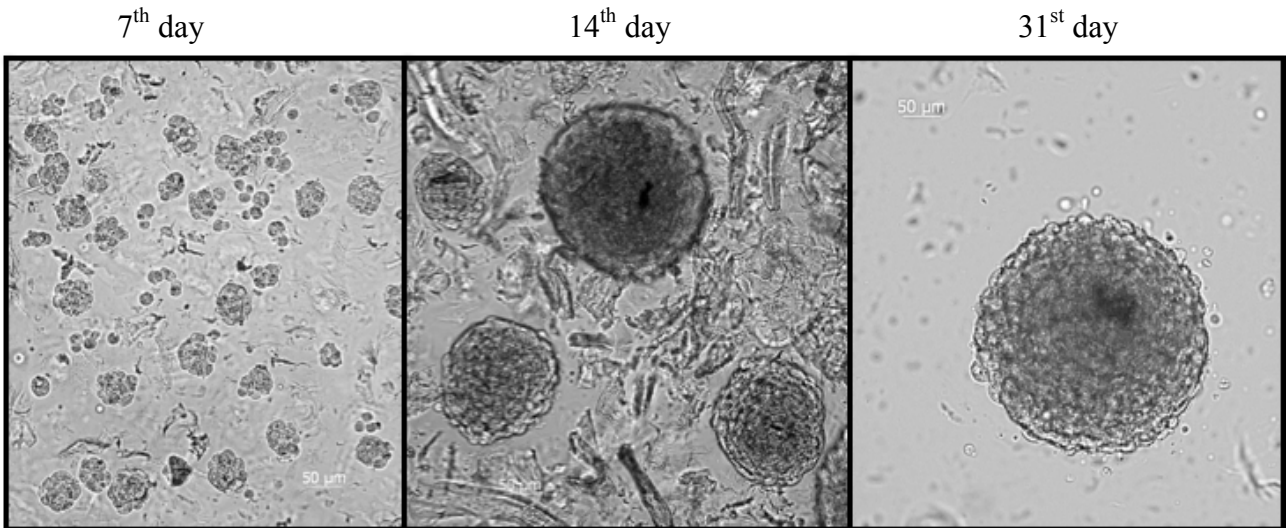
### **3.7. Determination of cell death type by Annexin V and PI assay**

Cells were seeded on the 6-well plate at the cell density of  $2 \times 10^5$  cells/well. Cells were treated with 30  $\mu\text{g}/\text{mL}$  and 40  $\mu\text{g}/\text{mL}$  of ascorbic acid and 10  $\mu\text{M}$  cisplatin. Control cells were untreated. All treatments were done in duplicates and incubated for 24 h. After incubation, the medium was transferred to 15 mL tubes, wells were washed with 1 mL PBS which was transferred to the tubes as well. 1 mL of trypsin was added and cells were incubated for 4 min at 37 °C and 5%  $\text{CO}_2$ . Trypsin was neutralized with 1 mL of GM. Everything from the wells was transferred to the tubes. Wells were washed with 2 mL of PBS which was transferred to the tubes. Tubes were centrifuged for 10 min at 400 x g and 4 °C. Tubes are kept on ice. The supernatant is aspirated and the pellet is resuspended in 1 mL of PBS. Tubes are again centrifuged for 10 min at 400 x g and 4 °C. The supernatant is aspirated and the pellet is resuspended in 1 x Annexin binding buffer (BD Pharmingen). 5  $\mu\text{L}$  of PI and Annexin V (BD Pharmingen) are added to each tube except the unstained sample. One sample contained only PI and one only Annexin V and were used for calibration of the flow cytometer.

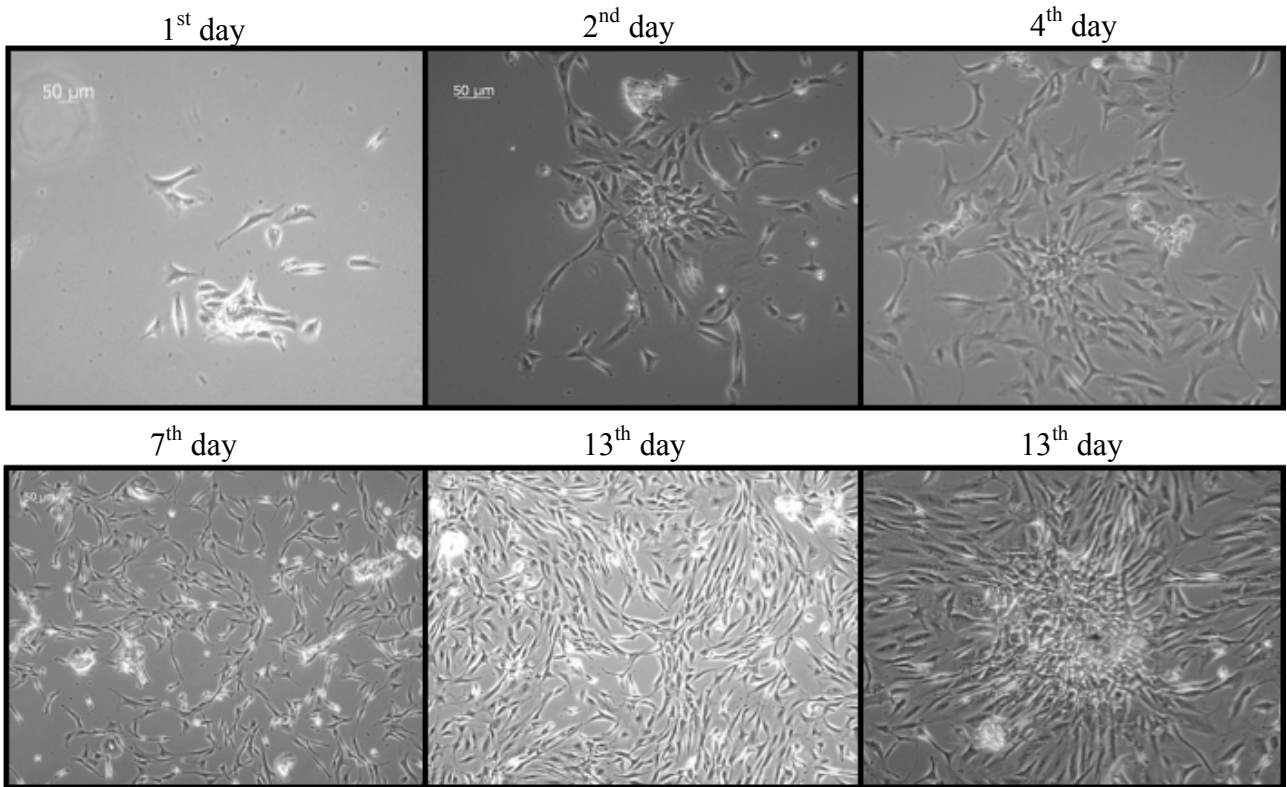
## **4. RESULTS**

### **4.1. Sarcosphere formation and adhesion**

OS-CSCs were isolated from patient's tumor and grown in growth medium as adherent culture. 40 000 cells/well was transferred in sarcosphere growth medium in 1% methyl-cellulose with presence of EGF and bFGF. Growth factors were added every three days to maintain the concentration of 10 ng/mL. OS-CSCs start as a single cell, dividing and clumping together forming a larger, oval structures. Figure 4. shows the formation and growth of sarcospheres. On day 7, sarcospheres are small (50  $\mu\text{m}$  in diameter) consisting of 6-30 cells. After 14 days, they become larger, approximately 150-200  $\mu\text{m}$  in diameter, while after a month, they become around 300  $\mu\text{m}$  wide in diameter. After 31 days of growth, sarcospheres were transferred into a 40  $\mu\text{m}$  nylon cell strainer and washed with GM so that excess methyl-cellulose and single cells are washed away. After filtration, sarcospheres were transferred into tissue-culture treated plates and incubated in GM. After the isolation of the sarcospheres and seeding them under adherent conditions and presence of FBS, sarcospheres started attaching and spreading at the bottom of the plate forming a monolayer as seen on Figure 5.



**Figure 4.** Sarcosphere growth in the period of 31 days. After 7 days, they are approx. 50  $\mu\text{m}$  in diameter and over the period of 1 month they grow up to 300  $\mu\text{m}$  wide, mag. 400x

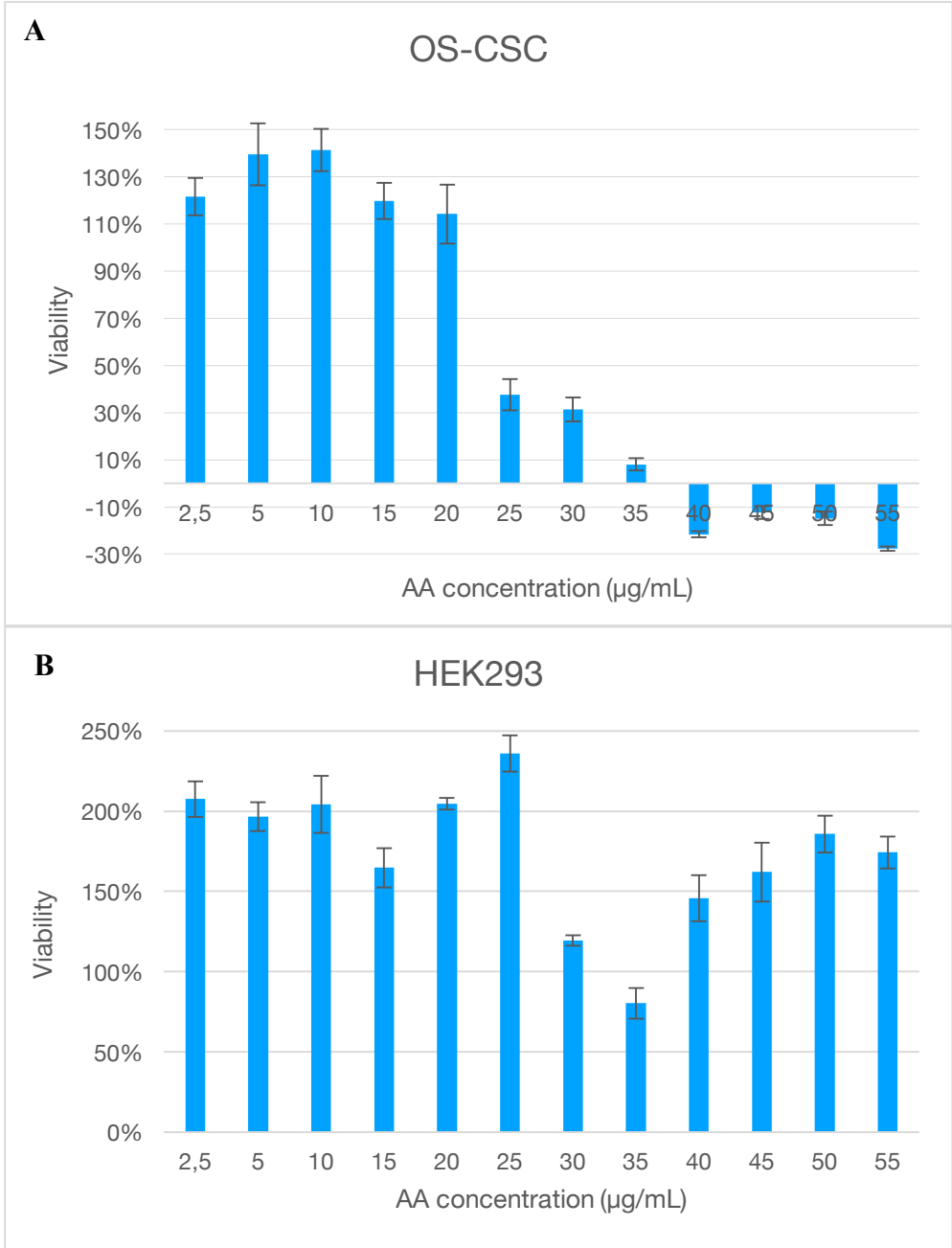


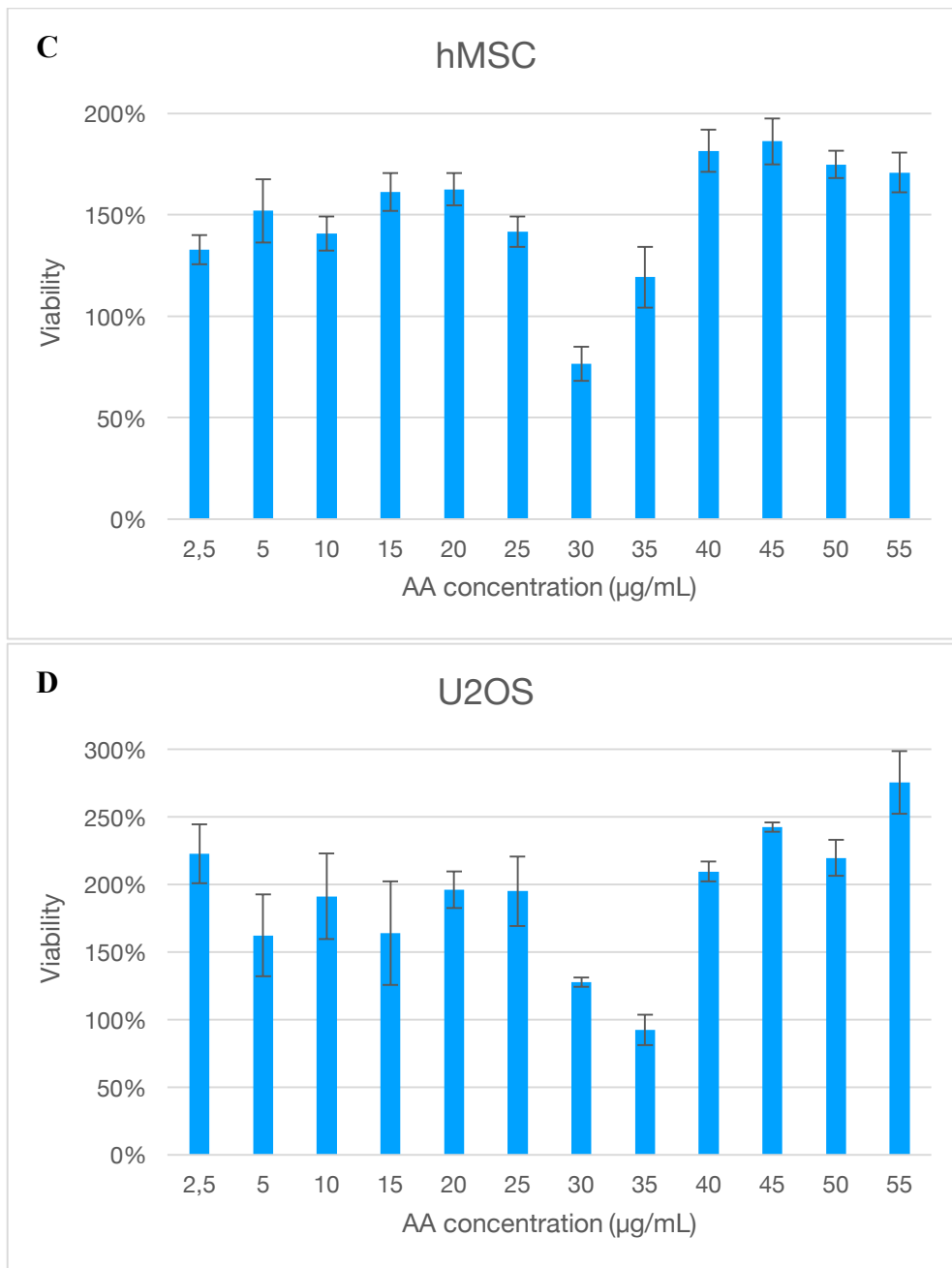
**Figure 5.** OS-CSCs adhesion and monolayer formation from sarcospheres, mag. 400x



## 4.2. Ascorbic acid effect on cell viability

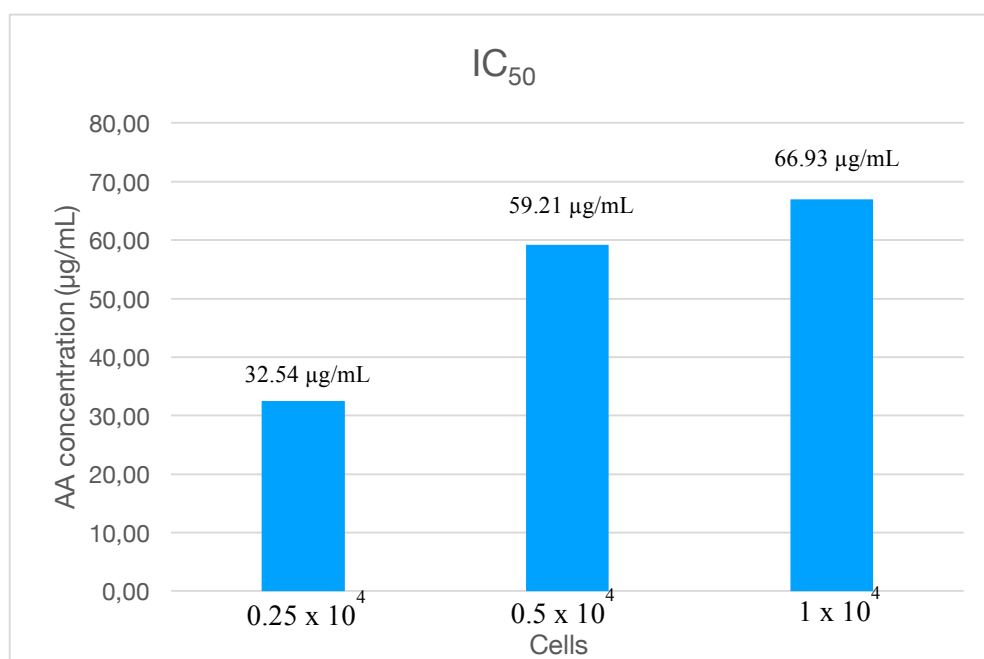
MTT assay was conducted to see the effects of AA on OS-CSC viability, to compare it to HEK293, hMSC and U2OS cell lines and to determine the concentration of AA that kills 50% of the cell population ( $IC_{50}$ ) (Fig. 6). Cells were treated with 12 different concentrations of AA (from 2.5  $\mu\text{g}/\text{mL}$  to 55  $\mu\text{g}/\text{mL}$ ) and incubated for 72 h. Cell density was  $0.25 \times 10^4$  cells/well. Viability of each sample is determined in comparison with control cells.  $IC_{50}$  is calculated and for OS-CSC it is 32.54  $\mu\text{g}/\text{mL}$ . The rest of the cell lines do not show viability less than 100% except in concentration of 35  $\mu\text{g}/\text{mL}$  AA. Concentrations 2.5  $\mu\text{g}/\text{mL}$ , 10  $\mu\text{g}/\text{mL}$ , 15  $\mu\text{g}/\text{mL}$  and 20  $\mu\text{g}/\text{mL}$  do not cause a big difference in cell viability as it is above 100% comparing to control cells. Concentrations 25  $\mu\text{g}/\text{mL}$ , 30  $\mu\text{g}/\text{mL}$  and 35  $\mu\text{g}/\text{mL}$  cause a significant decrease in cell viability as the viability drops below 50% in each sample. AA concentrations of 40  $\mu\text{g}/\text{mL}$ , 45  $\mu\text{g}/\text{mL}$ , 50  $\mu\text{g}/\text{mL}$  and 55  $\mu\text{g}/\text{mL}$  are killing all the cells in the samples. The viability of those samples is below 0%. HEK293 cell line shows increase in viability in samples treated with 2.5  $\mu\text{g}/\text{mL}$ , 10  $\mu\text{g}/\text{mL}$ , 15  $\mu\text{g}/\text{mL}$ , 20  $\mu\text{g}/\text{mL}$ , 25  $\mu\text{g}/\text{mL}$ , 30  $\mu\text{g}/\text{mL}$ , 40  $\mu\text{g}/\text{mL}$ , 45  $\mu\text{g}/\text{mL}$ , 50  $\mu\text{g}/\text{mL}$  and 55  $\mu\text{g}/\text{mL}$  concentrations of AA. The only drop in viability is recorded in sample treated with 35  $\mu\text{g}/\text{mL}$  (80.21% viability comparing to control group). hMSC cell line shows increase in viability in samples treated with 2.5  $\mu\text{g}/\text{mL}$ , 10  $\mu\text{g}/\text{mL}$ , 15  $\mu\text{g}/\text{mL}$ , 20  $\mu\text{g}/\text{mL}$ , 25  $\mu\text{g}/\text{mL}$ , 35  $\mu\text{g}/\text{mL}$ , 40  $\mu\text{g}/\text{mL}$ , 45  $\mu\text{g}/\text{mL}$ , 50  $\mu\text{g}/\text{mL}$  and 55  $\mu\text{g}/\text{mL}$  concentrations of AA. The only drop in viability is recorded in sample treated with 30  $\mu\text{g}/\text{mL}$  (76.58% viability comparing to control group). U2OS cell line shows increase in viability in samples treated with 2.5  $\mu\text{g}/\text{mL}$ , 10  $\mu\text{g}/\text{mL}$ , 15  $\mu\text{g}/\text{mL}$ , 20  $\mu\text{g}/\text{mL}$ , 25  $\mu\text{g}/\text{mL}$ , 30  $\mu\text{g}/\text{mL}$ , 40  $\mu\text{g}/\text{mL}$ , 45  $\mu\text{g}/\text{mL}$ , 50  $\mu\text{g}/\text{mL}$  and 55  $\mu\text{g}/\text{mL}$  concentrations of AA. The only drop in viability is recorded in sample treated with 35  $\mu\text{g}/\text{mL}$  (92.32% viability comparing to control group). Concentrations of AA used for further experiments are 30  $\mu\text{g}/\text{mL}$  and 40  $\mu\text{g}/\text{mL}$  to exclude the variability of cytotoxicity due to cell number variability.





**Figure 6.** Results of the MTT viability assay after 72 h. Twelve different concentrations of AA were used, ranging from 2.5 to 55 µg/mL. Viability was determined for 4 different cell lines: osteosarcoma stem cells (**A**), human embryonic kidney cells (**B**), human mesenchymal stem cells (**C**), and human bone osteosarcoma cells (**D**). IC<sub>50</sub> for OS-CSC is 32.54 µg/mL.

For determination of dependence of cell number on  $IC_{50}$ , cells were seeded on 96-well plate at three different seeding densities -  $0.25 \times 10^4$  cells/well,  $0.5 \times 10^4$  cells/well and  $1 \times 10^4$  cells/well. After seeding, cells were incubated for 24 h for them to attach to the bottom of the plate. After that, AA has been added in concentrations ranging from 2.5 to 55  $\mu\text{g/mL}$ . Cells were incubated 72 h. As it can be seen on Figure 7. the  $IC_{50}$  of AA increases with cell number. For the cell density of  $0.25 \times 10^4$  cells/well,  $IC_{50}$  was 32.54  $\mu\text{g/mL}$ , for the  $0.5 \times 10^4$  cells/well,  $IC_{50}$  was 59.21  $\mu\text{g/mL}$  and for the  $1 \times 10^4$  cells/well,  $IC_{50}$  was 66.93  $\mu\text{g/mL}$ .

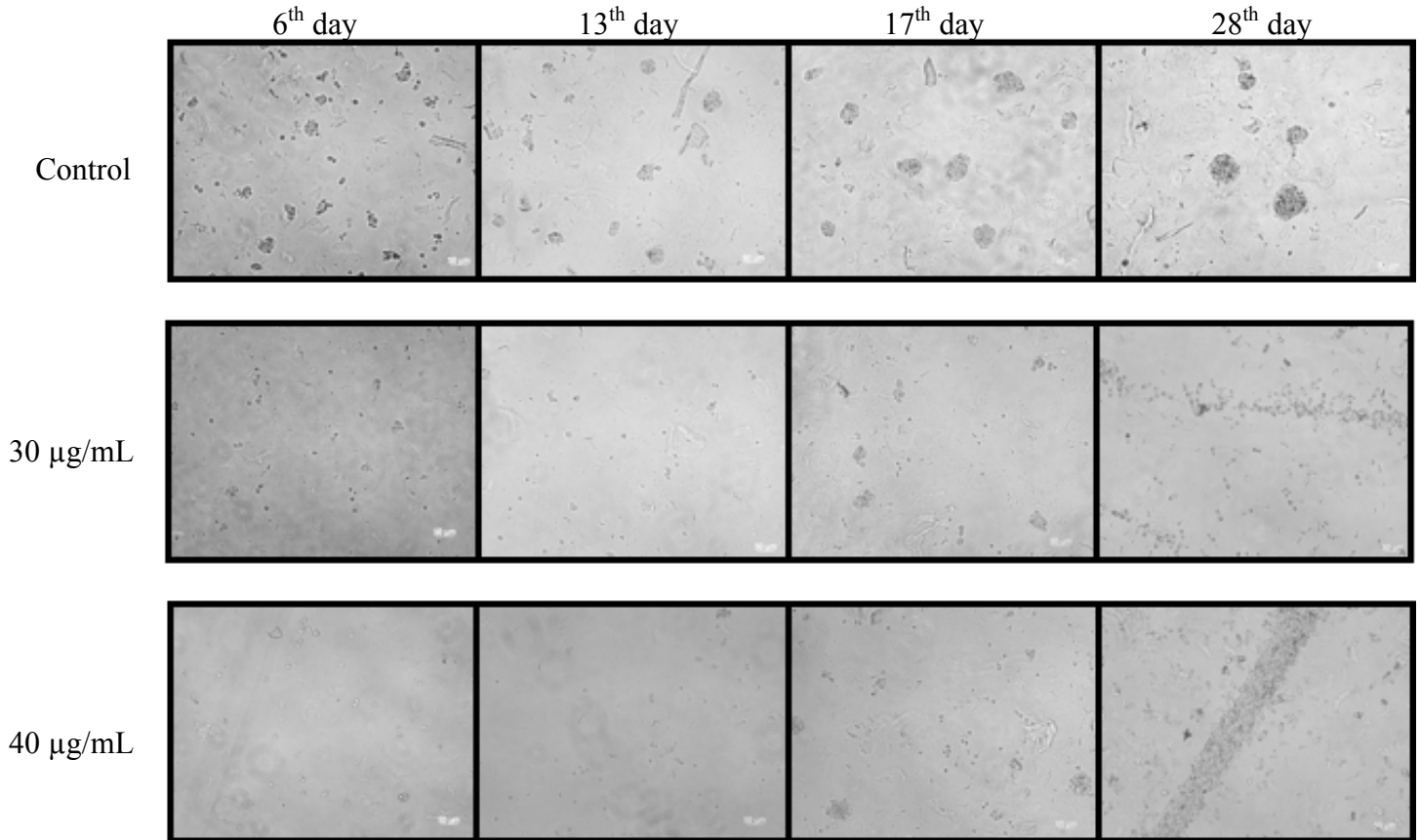


**Figure 7.** Correlation of number of seeded cells and  $IC_{50}$  of AA. OC-CSCs were seeded and treated with different concentrations of AA and incubated for 72 h.

### 4.3. Sarcosphere growth inhibition in the presence of AA

OS-CSCs were seeded under the low-attachment conditions and treated with 30  $\mu\text{g/mL}$  and 40  $\mu\text{g/mL}$  concentrations of AA. In Fig. 8 are shown photographs of sarcospheres in the period of 28 days. It can be seen that in samples treated with AA only several sarcospheres formed between 13<sup>th</sup> and 17<sup>th</sup> day, but by the 28<sup>th</sup> day, cells died. Control cells were not treated with AA. Formation of sarcospheres was used as a method to isolate OS-CSCs and this test was done to see if AA has the effect on viability of OS-CSCs in different, low-attachment conditions. Since the assay was done over the period of 28 days, AA needed to be added every

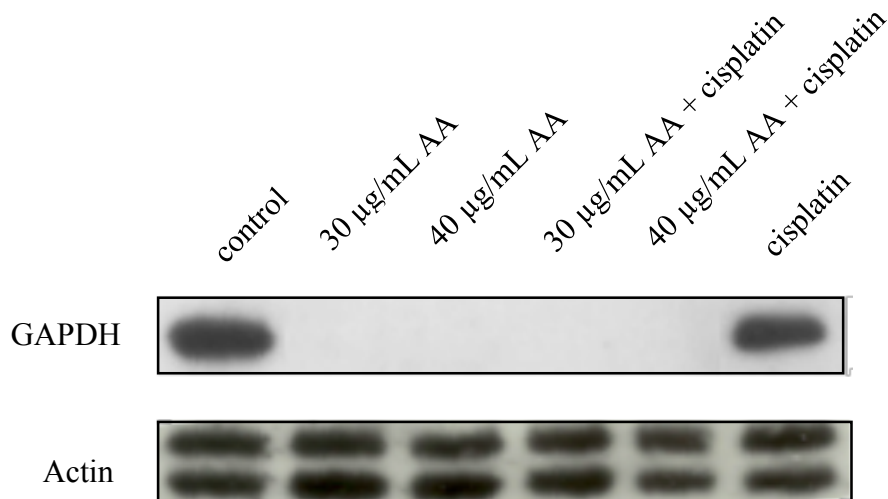
3 days to maintain the concentration. 0.0048  $\mu\text{g}/\text{cell}$  and 0.192  $\mu\text{g}/\text{cell}$  are the amounts of AA calculated from the MTT assay for the concentrations of 30  $\mu\text{g}/\text{mL}$  and 40  $\mu\text{g}/\text{mL}$ , respectively.



**Figure 8.** Growth inhibition of sarcospheres. 40 000 cells/well was seeded. Shown here are 6<sup>th</sup>, 13<sup>th</sup>, 17<sup>th</sup> and 28<sup>th</sup> day of sarcosphere growth inhibition assay. Concentrations of AA used are 30  $\mu\text{g}/\text{mL}$  and 40  $\mu\text{g}/\text{mL}$ , mag. 100x

#### 4.4. Effects of AA on glycolytic cycle

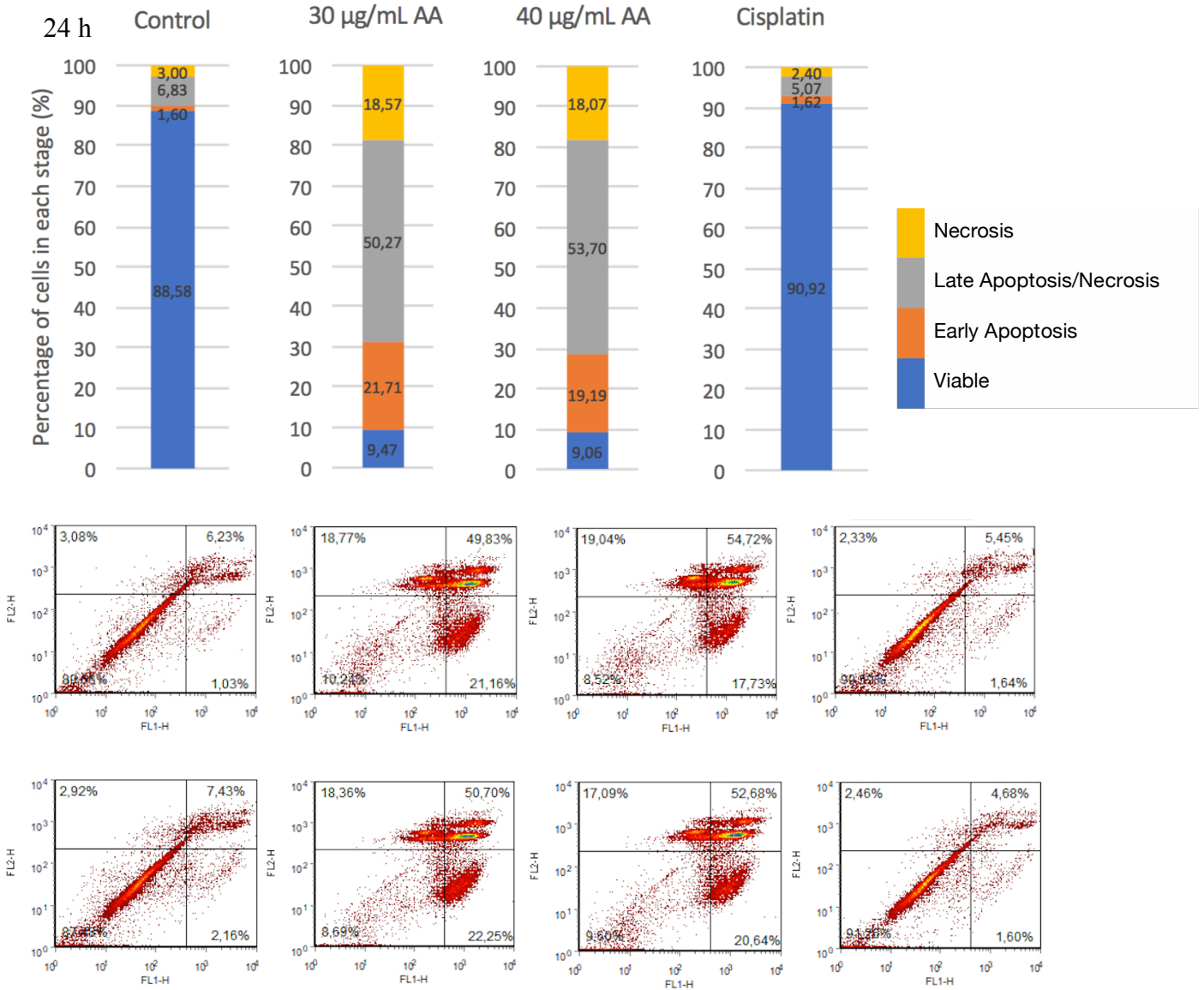
Western blot analysis was used to determine the levels of GAPDH in OS-CSCs treated with and without AA. Representative results are shown in Fig. 9. GAPDH is an enzyme that catalyzes sixth step of glycolysis and serves to break down glucose for the generation of ATP. Control cells were not treated with AA and show normal levels of GAPDH, as well as cells treated with cisplatin. In cells treated with 30  $\mu\text{g}/\text{mL}$  AA, 40  $\mu\text{g}/\text{mL}$  AA, 30  $\mu\text{g}/\text{mL}$  AA + cisplatin and 40  $\mu\text{g}/\text{mL}$  + cisplatin levels of GAPDH protein are undetectable. Actin was used as loading control.



**Figure 9.** Western blot analysis of GAPDH protein levels (5 µg total protein) in OS-CSCs. Cells are treated with 30 µg/mL and 40 µg/mL AA alone and in combination with cisplatin for 72 h. Actin is used as loading control.

#### 4.5. Determination of cell death type

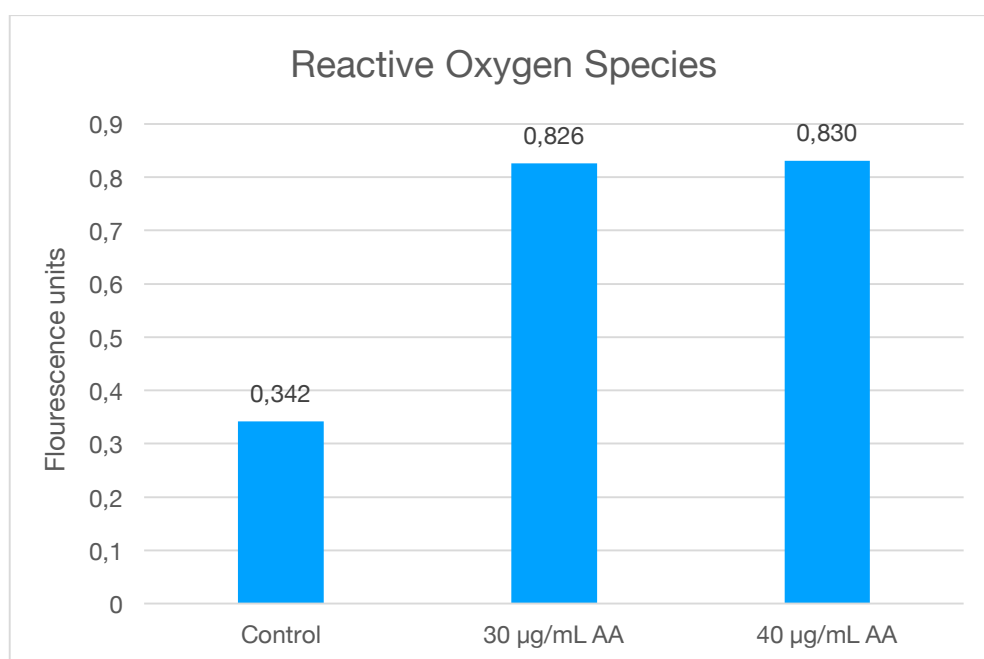
Flow cytometry was used to determine the cell death type caused by AA. Annexin V binds to phosphatidylserine that flips from inner to outer leaflet of the plasma membrane in cells that enter apoptosis and in that way, labels the cells. On the other hand, PI binds to DNA but cannot cross the plasma membrane which means it will bind only to cells with compromised integrity of the membrane. 30 µg/mL and 40 µg/mL concentrations of AA were used and 10 µM concentration of cisplatin. Cells were treated for 24 h. Graphs show average values of two independent treatments. Results are shown in Fig. 10. Control cells were mostly viable (88.58%), while a smaller portion was in early apoptosis (1.60%), late apoptosis/necrosis (6.38%) and necrosis (3.00%). Cells treated with 30 µg/mL were mostly in late apoptosis/necrosis (50.37%). Only small part of the cells remained viable (9.47%). In early apoptosis was 21.71% of the cells, and in necrosis 18.57%. Cells treated with 40 µg/mL showed similar distribution as cells treated with 30 µg/mL. 9.06% were viable, 19.19% in early apoptosis and 53.70% in late apoptosis/necrosis. In necrosis was 18.07% of the cells. Cells treated with cisplatin were 90.92% viable, 1.62% in early apoptosis, 5.07% in late apoptosis/necrosis and 2.40% in necrosis.



**Figure 10.** Annexin V/PI flow cytometry assay for cell death type determination. Graphs show average values of two independent experiments, percentage of cells out of all in the sample. Control cells were not treated. 30 µg/mL and 40 µg/mL concentrations of AA were used and 10 µM concentration of cisplatin. Cells were treated for 24 h.

#### 4.6. The effects of AA on redox state of OS-CSCs

This assay was done to examine the effects of AA on the amount of reactive oxygen species in OS-CSC. Cells were incubated for 72 h with 30  $\mu\text{g}/\text{mL}$  and 40  $\mu\text{g}/\text{mL}$  concentrations of AA. The results are normalized to the number of PI negative cells. PI negative cells include population of live cells and cells in early apoptosis, because the integrity of their membranes is still intact. Control cells show basal levels of ROS, and compared to them it can be seen that osteosarcoma stem cells treated with 30  $\mu\text{g}/\text{mL}$  AA and 40  $\mu\text{g}/\text{mL}$  AA show 2.44 increase in fluorescence intensity which means they have 2.44 times more ROS (Fig. 11). These results are suggesting pro-oxidative effect of AA in osteosarcoma stem cells.



**Figure 11.** Results of the ROS fluorometric assay. Control cells were untreated and used as basal level of ROS in the cell. Cells were incubated for 72 h with 30  $\mu\text{g}/\text{mL}$  and 40  $\mu\text{g}/\text{mL}$  concentrations of AA. The results are normalized to the number of PI negative cells.



## 5. DISCUSSION

After isolation of sarcospheres from the patient's tumor, they were successfully transferred from adherent to low-attachment conditions. Incubating them with growth factors, EGF and bFGF, in low-attachment conditions resulted in them forming sarcospheres. MTT assay was done to confirm selectivity of AA to OS-CSCs, determine if cell density affects  $IC_{50}$  and to determine  $IC_{50}$  for OS-CSCs. Selectivity is confirmed,  $IC_{50}$  for OS-CSCs is 32.54  $\mu\text{g/mL}$  for the cell density of  $0.25 \times 10^4$  cells/well, 59.21  $\mu\text{g/mL}$  for the cell density of  $0.5 \times 10^4$   $\mu\text{g/mL}$  and 66.93  $\mu\text{g/mL}$  for the cell density of  $1 \times 10^4$  cells/well. AA inhibited the formation of sarcospheres and GAPDH levels in cells treated with AA were not detectable. Flow cytometry showed that type of death caused by AA is either necrosis and/or apoptosis. ROS levels in cells treated with AA are elevated and the results suggest that AA is acting as a pro-oxidant.

As mentioned before, CSCs have the ability to form sarcospheres under low-attachment conditions. This property of OS-CSCs was used to isolate them from the tumor mass. Reynolds and Weiss (1992) were the first to describe the establishment of CSCs as spheres. Palmiini et al. (2016) determined that 40 000 cells/well is optimal number of cells to maintain isolation between them at the beginning of the experiment. It is crucial to avoid the possibility that the sarcospheres formed due to cell aggregation. Single CSC has the ability to grow under low-attachment conditions and form a spherical colony. After the cells were transferred to adherent conditions, they attached to the bottom of the plate and started spreading, forming a monolayer. The process of forming sarcospheres was repeated a couple of times and each time the cells successfully formed sarcospheres as well as monolayer. With that, we can conclude that the isolated cells really are OS-CSCs. Targeting CSCs is important as they are responsible for tumor relapse. There are 2 strategies for eradicating CSCs. Induction of differentiation forces CSC to become differentiated cancer cells which can then be targeted with conventional therapeutics. Inhibition of self-renewal insures inability of CSCs to divide and thus forcing them in apoptosis (Wang et al., 2015).

MTT viability assay was done to determine the  $IC_{50}$  value of ascorbic acid. Furthermore, the assay was done on three more cell lines to prove selectivity to OS-CSCs and to see if there is a difference in  $IC_{50}$  values if the cell density is higher. Selectivity is confirmed which can be seen from the data collected. HEK293, hMSC and U2OS cell lines did not have viability levels lowered by the increased concentrations of AA while OS-CSCs showed an increase in mortality with the increased concentrations of AA. Concentrations from 2.5  $\mu\text{g/mL}$  to 20  $\mu\text{g/mL}$  do not cause a big difference in cell viability as it is above 100% comparing to control cells.

Concentrations 25  $\mu\text{g/mL}$ , 30  $\mu\text{g/mL}$  and 35  $\mu\text{g/mL}$  cause a significant decrease in cell viability as the viability drops below 50% in each sample. AA concentrations from 40  $\mu\text{g/mL}$  to 55  $\mu\text{g/mL}$  are killing all the cells in the samples. The viability of those samples is below 0%. Considering the dependence of the cell density on the  $\text{IC}_{50}$  value, it can be seen that the more cells are seeded the higher the  $\text{IC}_{50}$  value is. With the cell density of  $0.25 \times 10^4$  cells/well,  $\text{IC}_{50}$  is 32.54  $\mu\text{g/mL}$ , for the  $0.5 \times 10^4$  cells/well,  $\text{IC}_{50}$  is 59.21  $\mu\text{g/mL}$  and for the  $1 \times 10^4$  cells/well,  $\text{IC}_{50}$  is 66.93  $\mu\text{g/mL}$ . The reason behind this changeable  $\text{IC}_{50}$  might be because every cell uptakes certain amount of AA and the more cells there are, the more AA is needed for them to die. This needs to be taken into account for further experiments. AA also inhibited the formation of sarcospheres. Xu et al. (2017) reported that SATB2 plays an important role in regulating OS-CSC properties and tumor growth. They isolated OS-CSCs by sarcosphere formation assay and conducted sarcosphere inhibition assay as well. Knockdown of the SATB2 disabled CSC properties *in vitro*, evidenced by the reduction of sarcosphere formation and cell proliferation. For further studies, it should be considered to test the combined effects of AA and SATB2 targeted therapies. Wu et al. (2015) showed that curcumin impairs tumor sphere formation of NCI-H460 lung cancer cells *in vitro*. Their results suggested that spheres reduced in size in a dose-dependent manner, the opposite of traditional chemotherapy drug Taxol, which could not decrease tumor spheres.

To try to understand the mechanism of action by which AA induces cell death, we conducted a series of experiments. To see if AA affects glycolytic cycle, we measured the level of GAPDH using western blot. It can be seen that the levels of GAPDH in cells treated only with AA and with AA in combination with cisplatin are not detectable. Another interesting result is that cisplatin alone did not cause any down-expression of GAPDH. Yun et al. (2015) tested if AA selectively kills KRAS and BRAF mutant colorectal cancer cells by targeting GAPDH. They investigated the mechanism based on the changes in metabolomic profile following the treatment with AA. Glycolytic intermediates upstream of GAPDH started accumulating, while the ones downstream depleted. Also, oxidative pentose phosphate pathway (PPP) metabolites increased which indicates that the GAPDH blockage causes shift from the glycolytic flux to the oxidative-PPP flux.

Another method used for clearing up the mechanism of action was the determination of reactive oxygen species in cells treated with AA. ROS levels in OS-CSCs treated with AA showed 2.44 times increase in respect to untreated cells. The reason behind the increase of ROS could be the decreased levels of NADPH/NADP<sup>+</sup>. NADPH/NADP<sup>+</sup> decrease activates glucose-6-phosphate dehydrogenase (G6PD) to enhance oxidative-PPP flux. Continuing, as DHA

uptake (GLUT-mediated) depletes cellular glutathione (GSH) and NADPH, as they are needed in reducing DHA in AA, capacity of the GSH restoration pathway is exceeded and ROSs begin to accumulate (Yun et al., 2015). Schoenfeld et al. (2017) reported that in non-small-cell lung cancer (NSCLC) and glioblastoma (GBM) GLUT-mediated DHA demonstrated a minimal role in AA toxicity. Their results suggested that because of increased mitochondrial  $O_2^-$  and  $H_2O_2$ , levels of labile iron pool (LIP) increased as well. Their theoretical model suggests that increased LIP in cancer cells results in increased oxidation of AA that generates  $H_2O_2$  capable of further worsening the difference in labile iron in cancer cells as oppose to normal cells. Increase in  $H_2O_2$  in the presence of increased labile iron results in generation of hydroxyl radicals causing oxidative damage. Furthermore, De Francesco et al. (2015) developed a synthetic lethal strategy for eradication of CSCs. They first reduced cellular respiration and targeted mitochondrial protein translation with doxycycline and with that metabolically synchronized the surviving cancer cell sub-population to predominantly use glycolysis. Following, treatment with AA blocks the glycolysis by inhibition of GAPDH and successfully eradicates CSCs.

Flow cytometry was used to determine which type of cell death is triggered by AA. Control cells and cells treated with cisplatin do not show any signs of reduced viability which was expected and confirms that OS-CSC are resistant to conventional chemotherapeutics due to its high activity of ABC transporters. Cells treated with AA are mostly in late apoptosis/necrosis. There is a population of cells in necrosis and early apoptosis which suggests that certain portion of the cells dies through apoptotic pathway. Mata et al. (2015) reported from different sources that AA can activate apoptosis, necrosis and aponecrosis. Further studies should be conducted to see if there are one or more death types caused by AA.

## 6. CONCLUSION

CSCs have the ability of self-replication and differentiation in different cell types which ensures them tumorigenicity. Active membrane pumps and repair enzymes provide them resistance to conventional chemotherapy. Osteosarcoma is a malignant tumor and development of selective therapies for OS-CSCs eradication is necessary to improve patient survival. The goal of this study was to investigate the effects of AA on OS-CSCs.

Based on the obtained results, it can be concluded that AA selectively kills OS-CSCs. It prevents formation of spheroids and its  $IC_{50}$  is dependent on the number of cells. The mechanism of death is based on the blockage of glycolytic cycle and increased intracellular levels of ROS which triggers either apoptosis and/or necrosis.

This research provides an insight into the effects of AA on OS-CSCs. Targeting CSCs in tumor is necessary to eradicate the tumor and this research showed that AA, indeed, selectively kills OS-CSCs.

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# CURRICULUM VITAE

I was born on March 28<sup>th</sup>, 1994, in Zagreb, Croatia. I completed my high school education in my hometown. In 2012, I enrolled in the Undergraduate program of Molecular biology at the Faculty of Science, University of Zagreb. I completed laboratory practice under supervision of Dubravko Pavoković, Asst. Prof., at the Division of Molecular Biology and received a Rector's Award. During my final year of undergraduate studies, I was part of the 9<sup>th</sup> International Society for Applied Biological Sciences (ISABS) Conference on Forensic and Anthropologic Genetics and Mayo Clinic Lectures in Individualized Medicine. I was a teaching assistant for Zoology and Laboratory Animals in Biological Research courses. I graduated from the undergraduate program by writing a final paper titled «The role of hair in forensic analysis» under supervision of Inga Marijanović, Asst. Prof. and obtained a title of bacc. mol. biol.

In September of 2015, I enrolled in the Graduate program of Molecular Biology. I was a teaching assistant for Introduction to Forensic Biology, Molecular Genetics, Research Methods in Molecular Biology, Mutagenesis and Carcinogenesis, Biology of Stem Cells and Cell Biology courses. In 2017, I was part of the 10<sup>th</sup> ISABS Conference on Forensic and Anthropologic Genetics and Mayo Clinic Lectures in Individualized Medicine. I organized an event for popularization of science “Night of Biology” in 2013, 2014 and 2016. I spent 4<sup>th</sup> semester in Madrid, Spain as an Erasmus+ student. After returning to Zagreb, I started working on my Master's thesis under supervision of Inga Marijanović, Asst. Prof.