



Research Article

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Is it Possible to Expedite Studies on the Effects of Pharmacological Agents on Primary Cell Cultures Obtained from High-grade Fibular Osteosarcoma?

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Abstract

Cell culture and primary cell cultures have been widely used in research in many fields of medicine, particularly cancer research. With the help of cell culture studies, the underlying molecular mechanisms and effects of pharmacological agents have been determined to a great extent. The tumor tissue used for osteosarcoma primary cell culture was obtained from resection surgery carried out in cases with proximal fibula fibroblastic osteosarcoma after three multiagent (neoadjuvant) chemotherapy treatments. The present study not only describes how to prepare human primary osteosarcoma (OS) cell cultures but also encourages and guides orthopedic surgeons in how to set up an *in vitro* experiment for a drug research.

Keywords

Cytotoxicity research; Drug administration; Primary cell culture; Sarcoma of the fibula

Introduction

The use of cell cultures has been highlighted in recent studies about regenerative medicine. Like other areas of science, orthopedic surgery has focused on studies where experiment setups with cell samples are discussed, thereby integrating orthopedic surgery into cell culture research [1-10]. One of the research subjects of these studies is osteosarcoma (OS), which is generally seen around the knee in people under 30 years of age, affecting approximately 1,000 people in Europe and the United States [11,12]. In contrast to carcinomas, primary bone sarcoma is quite rare. Furthermore, the number of experimental and clinical studies in this field has been limited. As a result of extensive research concerning treatments of tumors based on carcinoma and hematogenous tumors, there have been significant enhancements in the survival of cases over the past 10 years. However, no significant

pharmacologic agents or new clinical treatments in sarcoma chemotherapy have been introduced in the past 30 years. Most cancer-oriented drugs that have been approved or are now being researched have side and/or adverse effects because of toxicity; moreover, the therapeutic dose-response curve has not been comparatively studied at the cell level with consideration of the specific characteristics of the case. Moreover, there is a need to carry out further studies focusing on the genes of tumor carriers and different variations of genes that affect the tumor cells' resistance to drugs or genes encoding drug metabolism enzymes. This pharmacogenetics, pharmaceutical technology and pharmacomolecular/genetics studies will be essential to enhance the knowledge concerning the interaction of drug therapy; to this end, the use of cell cultures in *in vitro* experimental research is crucial [1-8].

One topic that should be studied is whether pharmaceutical agents imbued into various pharmaceuticals and drug carriers have a cytotoxic effect on OS to inhibit local relapse and metastasis despite wide resection and systemic chemotherapy and/or radiotherapy [12-17]. Different experimental setups are needed to examine the cytotoxicity of a drug [18-23]. However, proper cell proliferation and the appropriate cell microenvironment are essential when it comes to imitating the target tumor tissue. For this very reason, cells with the appropriate morphology should be cultured *in vitro*. The present study aims to introduce a primary culture model that orthopedic oncologists and sarcoma clinicians can apply in practice. The goal is not to present how to prepare human primary OS cell cultures but rather to encourage orthopedic surgeons to set up *in vitro* cell experiments by means of brief information concerning cell isolation, culture techniques and characterization.

Materials and Method

This scientific research study was approved by the ethics committee. In addition, all of the participants provided written informed consent for their tissue materials to be obtained. During and after the research, the Declaration of Helsinki criteria were adopted.

Materials

Essential equipment and hardware: magnetic stirrer, microbalances, laminar air flow, a class 2 biosafety sterilization system, distillate and ultrapure water treatment systems, a shaking hot water wash with adjustable heat, a cooled centrifugal machine, a fridge, a cell count checker, and various pipettes from 1 to 1,000 µl. In addition, pipette tips of various sizes, culture petri dishes with an area of 60-100 mm area), pastor pipettors, autoclave-durable metal boxes, and 15 and 50 cc falcon tubes were needed.

Catling and bistoury equipment was needed to mince tissues mechanically. In addition different well plates and flasks with pores that would enable gas input/output with a swan shape were needed.

The following additional equipment was necessary: insulin, transferrin, and a premix solution composed of selenious acid (ITS); Dulbecco's Modified Eagle Medium (DMEM); Roswell Park Memorial Institute (RPMI)-1640 medium; inactivated fetal bovine serum (FBS); penicillin-streptomycin (PS) and trypsin-ethylene diamine tetraacetic acid (EDTA) solutions; phosphate tampon solution (PBS);

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and dimethyl sulfoxide (DMSO). Collagenase enzymes of *Clostridium histolyticum* origin were also needed. Moreover, 0.2 µm diameter pores to be used in percolation were needed before PBS was added to distilled water or FBS was added to the cell culture environment.

Methods

Eligibility criteria: Before setting up cell cultures, clinical, radiological, and pathological OS cases involving difficult diagnoses should be evaluated by professionals who are experts in this field. During pathological evaluation, the pathologic grade report in the World Health Organization classification should be considered, as well as the typical histological appearance of OS [24]. It is important that cases with poor diet, serious nephropathy and/or hepatic failure, AIDS, bone marrow hypoplasia, leucopenia, thrombocytopenia, anemia, and alcohol addiction, as well as cases who are pregnant or allergic to egg or drugs are excluded from the study.

Surgical technique in a sample case and preparation of primary cell culture: A 13-year-old female with located lytic destructive bone lesion in the proximal fibula and coexisting soft tissue mass underwent core-needle biopsy after scanning the tumor (Figure 1). Following the biopsy results, the case was diagnosed with high-grade (grade 3) fibroblastic type OS, and three preoperative treatments were administered using the multiagent OS protocol. Next, the “en bloc” procedure was applied in the proximal fibula with tibiofibular articulation through the tumor-invasive peroneal motor nerves. The resection contained an approximately 12-cm segment (Figure 2). The area that was resected after surgery corresponded to the fibula neck, which was soft tissue mass. The muscle cover was removed and sampling of 1×0.5 cm of tissue was carried out. Sign sutures were placed in this area and the muscle cover was lifted. The remainder of the resected tissue was sent for pathological examination in formol solution. After the pathological examination of the specimen, 30% chemonecrotic tissue was observed (70% living tumor), and an advanced, undifferentiated fibroblastic type OS was reconfirmed (Figure 3). The sampling obtained from resected tissue was transferred into medium. The transfer medium was prepared with 100 mL RPMI-1640 or DMEM contained 5 mL of PS (10,000 U/mL). Sterile, capped falcon tubes were transferred into the laboratory at 4°C in appropriate conditions (maximum duration: 2-6 h).

Preparation of primary osteosarcoma: Tissues were transferred into the laboratory in appropriate conditions and placed the laminar flow cabin. Five sterile petri dishes were used. Tissues were transferred to four petri dishes in 50 mL volumes through an irrigation process

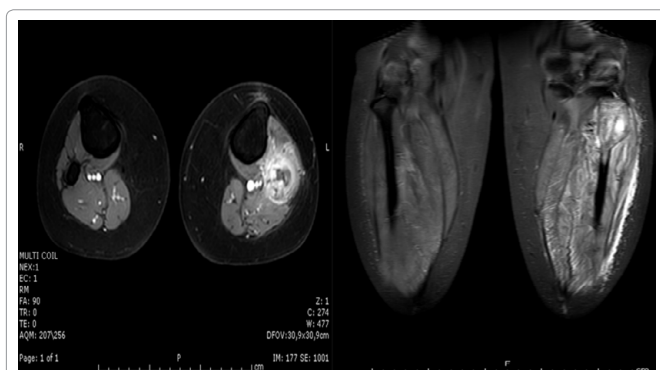


Figure 1: Sagittal and axial T2-weighted magnetic resonance images of proximal fibular osteosarcoma showing intramedullary infiltration and soft tissue extension of the tumor.

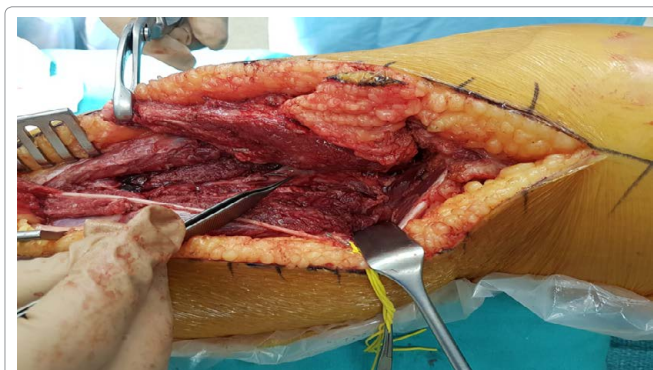


Figure 2: Intraoperative image of the wide resection of the proximal fibula, including healthy soft tissue margins, the deep peroneal nerve and peroneal vessels.

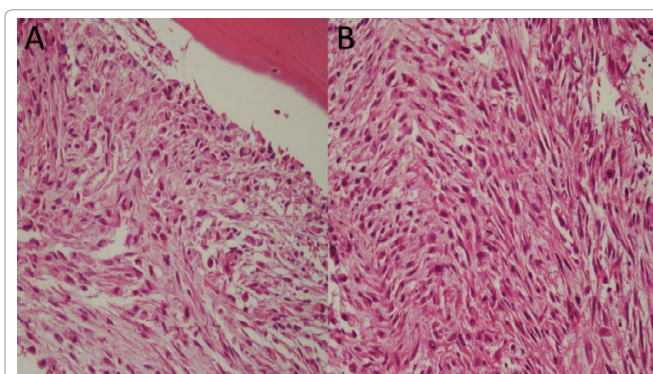


Figure 3 A-B: Microscopic view of high-grade fibroblastic osteosarcoma; biopsy (A) and (B) after neoadjuvant chemotherapy; hematoxylin and eosin; x40 magnification.

using pliers, with the irrigation process carried out to avoid red blood cells. Irrigation was performed using PBS cooled and adjusted to pH 7.4 or 0.9% isotonic sodium chloride solution. Tissues transferred into the fifth petri dish were minced with the help of a catling, pliers, and a rodent. Next, these tissues were worked up into 1–3 mm³ pieces and transferred into 15 mL falcon tubes with collagenase type I and/or type II enzymes; at this point, they were dissolved in Hank's Balanced Salt Solution.

After the tubes were closed tightly, they were placed into the incubator on racks; the incubator was adjusted to at 37.4°C with 5% CO₂. The falcon tubes were loosened enough to enable gas input and output and left in the incubator overnight. Following this, the falcon tubes were taken out of the incubator and closed tightly. Three cooled centrifuge processes were carried out consecutively at 1,200 rpm for 10 min, and then the supernatants were removed. Freshly prepared culture medium was added to cream-colored cell pellet (Figure 4). Gentle shaking was carried out with the help of micropipettors. Cells were placed in flasks in a swan-neck shape. These flasks allowed gas input and output and had a surface area of 25 cm². At least 15 mL of freshly prepared culture medium was added. Complete fresh medium prepared with 50 mL RPMI-1640 or DMEM contained 0.5 mL of PS (5,000 U/mL) and 1 mL of L-glutamine (200 mM), 1 mL of ITS, and 10% FBS [8].

Samples in flasks were kept in the incubator for 72 h. At the end of this period, the confluence rate and viability were controlled by means of inverted light microscopes. The culture medium was replaced every

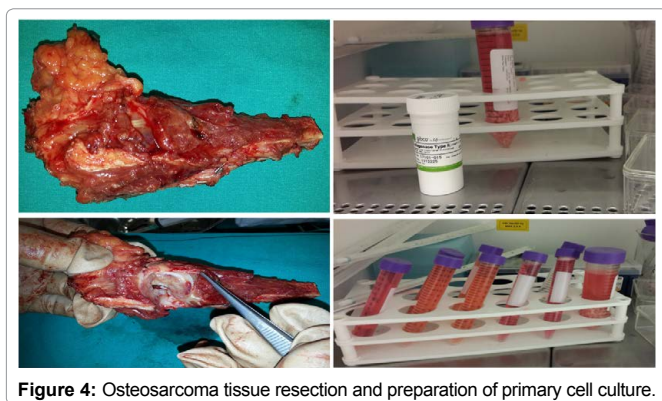


Figure 4: Osteosarcoma tissue resection and preparation of primary cell culture.

2 days. A proper culture medium contains the amino acids, glucose, growth factors, and vitamins necessary to maintain cell cultures. Moreover, the osmolarity and pH values of the culture environment should be appropriate for the characteristics of the cells [4,8,25]. It was necessary to add FBS and ITS to the primary OS culture medium environment with the purpose of obtaining healthy and proliferous cells with high confluence [4,8,25]. FBS was added as a serum because it contains intercellular matrix proteins to make it cling to the surfaces of flasks where cells were planted. In addition, the hormones, enzymes, and growth factors necessary for cell proliferation were provided by FBS [8]. The solution containing PS was needed to avoid effects from environment, which are evident in all normal primary cells. The mixture ratio should be 1% of the environment [1-8]. The medium of the samples in flasks was replaced with every 2 days.

Inverted light microscopy: Before starting the inverted light microscopy process, it was necessary to remove dust from the lenses of microscopes and eyepieces using 70% ethanol. Flasks were carefully placed on the microscope platform.

It was important to avoid contact between the necks of the flasks and the prepared culture medium. Initially, observation of how much of the flask surface was covered with cells was carried out. Micro-images at magnifications of 10 \times , 20 \times , and 40 \times needed to be recorded to evaluate how many cells cling to the bottom and whether they were healthy or proliferous.

Cells that had a logarithmic increment in the culture vessels where they were planted covered almost the entire surface and did not achieve production. They ought to be taken from the environment when their production is suppressed. This process, which called excerpption of cells, occurred under two harvesting methods. The first method was that they could be removed through a cell scraper without using chemicals. The second method was an exception process in which trypsin and/or collagenase was used in combination with EDTA (0.25). Using cell scraper may result in cell deformation [8]; therefore, use of the trypsin-EDTA mixture, which hindered proteolytic reaction, was more appropriate for the culturing of cells.

The crucial point here was that cell culture media content (CCMC) needed to be removed from the environment before the trypsinization process to separate cells from the bottom of the flasks. The flasks bottoms needed to be washed three times using PBS, since serum in CCMC contains trypsin inhibitors. Therefore, flasks needed to be washed at least once using PBS that did not contain calcium and magnesium. Next, the serum on the surface was removed from the environment.

Trypsin (with 25% EDTA content) was added according to the size of the petri dishes; 2 mL was added to 100 mm dishes, 1 mL to 60 mm dishes, and 0.5 mL to 35 mm dishes. They were then kept for 2 h in the incubator. Cells were left for 4 m for max in the incubator to verify that they had been removed from the surface using an invert microscope. This was done to ensure that the proteolytic reaction was complete. After removal of cells from the surface by trypsin, the proteolytic reaction should cease, as this may damage cell membranes. For this reason, 4 mL, 2 mL and 1 mL volumes of complete medium containing 10% FBS were added to inhibit the proteolytic reaction. The content was transferred to falcon tubes and the supernatant in the tubes was removed; the tubes were then centrifuged for 5 min at 1,200 rpm. Freshly prepared CCMC continued to be added to the pellet on the bottom.

Cell counting: A hemocytometer was used for cell counts. This was an analysis based on the principle that lamella was put on a lame, which has counting rooms. On the lame, there were squares whose lines were scraped at 1 mm. Within the squares were surface areas smaller than the squares. Cells were provided to be taken from culture vessels through trypsinization.

Suspended cells were homogenized at least 2 or 3 times using a micropipette. For excerpption to take place the surfaces of culture vessels need be entirely covered; in other words, they should be confluent. The application ratio was as follows: 2 mL of trypsin for a 100 mm petri dish, 1 mL trypsin for a 60 mm petri dish, and 0.5 mL of trypsin for a 35 mm petri dish.

To calculate the number of cells, 10 μ L of the diluted cell suspension in 1 mL of culture medium was placed in the Eppendorf tube. This was mixed by adding 90 μ L of Trypan blue dye to the suspension.

The mixture was put into a lame cap, so the number of cells in 1 mL of medium could be found after calculations were made. Following the calculation, plantation into a flask, well plate, or petri dish was carried out.

Trypan blue, one of viability colors, was used in culture conditions during the count process because it can easily penetrate through dead cell membranes and stain the cells. At the end of this process, it was possible to report the total number of dead cells per millimeter and the rate of living cells.

The result of the counting process in the lame was done by using the following formula: $N \times DF \times 16 \times 10,000$. The expressions were as follows: N=the number of cells in middle square; DF=the dilution factor; 16=a multiplier used to find all squares' result, as there were 16 squares; and 10,000=an invariable used to convert a count of 0.1 mm³ into 1 mL and to reach a standard result.

As the blue color could not penetrate into living cells, the images of living cells showed that they were small, retractile and had a rounder morphology compared to dead cells. The images of dead cells were different, as they were bigger than living cells and colored with thiazolyl blue.

Application of drug in cultured samples: Studies on vaccines, monoclonal antibodies, the production of various enzymes and hormones, measurement of inner cell activity, deoxyribose nucleic acid, and replication of deoxyribonucleic acid or energy metabolism have been made possible by means of cell culture studies. In addition to such research, it is possible to study various behaviors of receptor

complexes, signal transduction mechanisms, and the effects of various drugs on cell cycles. Furthermore, with the help of primary cell culture researches, it is possible to study viral transformation, chemical transformation, excretion of special products, embryonic, cell population kinetics, cytogenetics analysis, cytotoxicity, and genetic manipulation and immortalization, especially in relation to cancer treatments. Somatic gene treatment, tumor vaccines, using living vaccines in grafts, and the formation of three-dimensional (3D) tissues are among the promising studies regarding culture research.

When drugs are added to a culture environment, the researcher should not be aware of the groups in which the drug has been applied; in other words, s/he should be blind to the culture environment. To sustain this, another researcher, senior researcher, or the project coordinator should code the samples using numbers and letters after drugs have been placed light-proof bottles. Another key point is that, as in all research, not only the analyses but the entire experiment should be repeated at least three times. Such blinding and replication were used in the present study. After the drugs were placed into bottles and the coding process was completed, culture samples without any drug included were fed in an ordinary way with the purpose of forming the control group. Meanwhile, the study samples were given the designated doses of drug.

If the amount of a dose to be used in cell cultures had been reported in a reliable study, that dose could be used. If the dose was not known, a designated dose was taken from the drug in the flow cabin whose main stock was certain. The solubilized amount was recording to determine how many micrograms were used per milliliter (e.g., DMSO). From the solutions that were solubilized in appropriate dissolvent, diluted and homogenous solutions were prepared starting from 1,000 μM and moving to 500, 250, 100, 10 and 1 $\mu\text{M}/\text{mL}$.

Drug solution was added to the culture environment with consideration of the cell culture setting (e.g. 3 mL medium and 3 mL diluted drug solution). The same experimental setup had to be prepared for the six main groups and the control group, including spare cell classes. These setups were used in the analyses carried out at 0, 24, 48 and 72 h.

The cell viability, toxicity, and proliferation of the researched drug were examined. In addition, the same setup should be prepared for each analysis and period, if apoptosis and/or scanning electron microscopy (SEM) is done.

Scanning electron microscopy (SEM): Extra samples that had been prepared for SEM needed to undergo some processes to sustain cell fixation. To this end, 8% glutaraldehyde and cacodylate buffer solutions were needed. Glutaraldehyde was taken from the solution in a sterile laminar cabin and diluted by sterile water from 8% to 2.5% in direct proportion. To this end, 31.25 mL was taken from a glutaraldehyde solution and put into a measure. Next, distilled water was added to create a final volume of 100 mL. This solution was transferred to sterile glass bottles with screw vessels, and they were shaken until they became homogenized. Then, 97.5 ml was taken from cacodylate buffer solution, and 2.5% glutaraldehyde solution was added, and the vessels were shaken.

These prepared solutions were added to all plates with cells to cover the samples. The cells were left for 130 min, which was a length of time used in previous studies. Cacodylate buffer mixture with glutaraldehyde was removed from the environment using a pipettor gun. Following this, samples were washed three times using pure cacodylate buffer without glutaraldehyde. After the last wash, cacodylate buffer was added to the samples until they were covered. Next, they were left in the fridge at 4°C until they were transferred to SEM. In addition, they could be transferred to SEM analysis immediately after they were covered with aluminum foil. If an environmental scanning electron microscope was used instead of a scanning electron microscope, the samples could be scanned after the supernatant was taken, so there was no need to cover them using silver or gold stain (Figure 5).

Immunoflow cytometry: The immunoflow cytometric analysis method, based on the principle that they are identified by using monoclonal antibodies against antigens, can be used for clinical diagnosis or research targets. In this approach, marked, suspended cells arrive in the flow cabin by means of a high pressure system. Next,

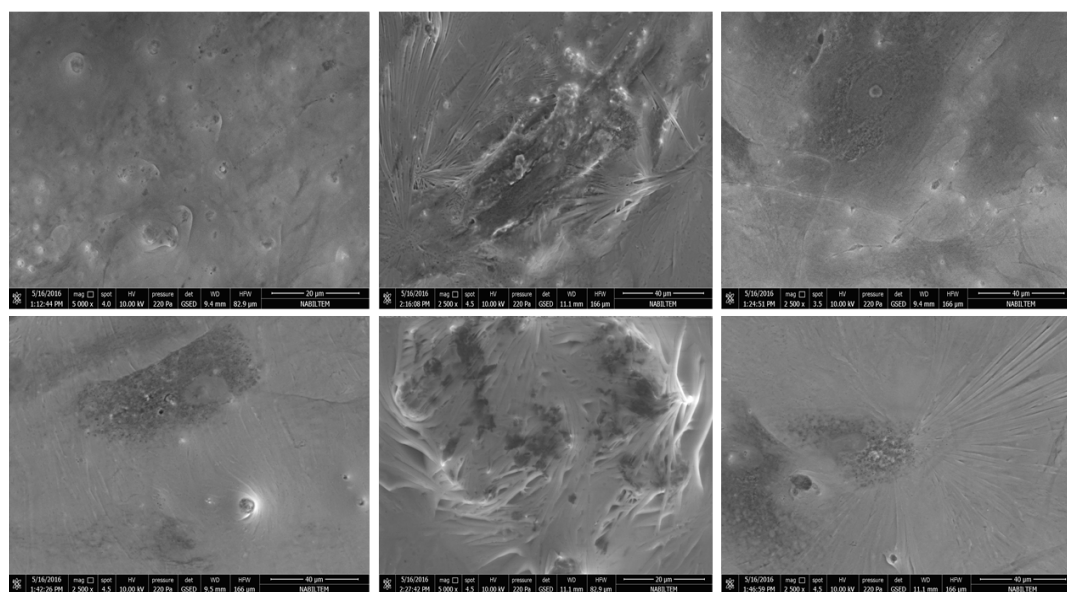


Figure 5: Evaluation of surface morphology belonging to osteosarcoma cells through ESEM.

they pass through laser beams one by one in different photodetectors. In addition, in the immunoflow cytometer, a stain, such as propidium iodide (PI), ethidium, Texas Red, fluorescein isothiocyanate (FITC), or phycoerythrin (PE) can be used. However, if the process is carried out using multicolored stains, false negatives or false positives may be detected.

Cells that clung to the bottom of the flask and had enough confluence in the inverted microscope evaluation were gently moved into falcon tubes containing a freshly prepared cell culture environment after they were excerperted by trypsin-EDTA or a scraper. The pellet on the bottom after the cooling (4°C) process, which was carried out three times for 5 min at 1,200 rpm, was resuspended by CCMC. Following the cell count (10^3 - 10^6), a stain, such as FITC-PE, was added to the cells that had been suspended by CCMC. Next, conjugate monoclonal antibodies were added and incubated for 50 min at 22.4°C (as cluster of differentiation-44 marker in our study). PBS, which was prepared with 0.1% sodium azide, was added to the environment and centrifuged for another 5 min at 1,200 rpm. The supernatant was removed from the environment. The resuspended cells were processed through the cytometer machine and evaluated using a software program.

Histopathological evaluation: In the laminar flow cabin, glass lames were placed in the wells in the well plates where seed of samples of cell cultures was carried out. Following this, samples were cultured, planted, and incubated extremely slowly and carefully in these wells in the lamella.

After incubation, cultured cells could be applied to the culture samples to reach the desired confluence by inverted light microscopy. For this reason, lames were placed in the cabin. Lames in the well were removed using a 21-gauge sterile injection needle while avoiding damage and closed upside down using forceps (Figure 6). Then, histopathologic preparates were prepared using stains.

Primary cell culture samples were taken between the lame and lamella with the help of a Pasteur pipette and treated by 96%, 80%, and 70% (weight/volume) ethanol. Samples were washed with tap water and kept in May-Grunewald solution for 1 min. At the end of this time, they were washed again with tap water to dispose of the stain. Then, they were kept at room temperature for 1 min. After they were kept in Giemsa solution for 2 min, they were washed again twice.

A closure solution, such as balsam, was used to close the samples taken between the lame and lamella. If hematoxylin and eosin stain was to be used, the same protocols were realized. However, unlike from Giemsa, where the preparates were washed twice by tap water for 1 min each after the wait time, these preparates were also washed by 96% ethanol (weight/volume). Afterwards, closure solution was used to close samples taken into between lame and lamella. Following these steps, samples were evaluated using a light microscope at magnifications of 4 ×, 10×, 20×, 40×, and 100× (Figure 7).

In the samples, there were cells with flat and irregular cytoplasm, big oval nuclei, thin chromatin structures, and eligible nucleoli. These cells were found to be compatible with active fibroblasts. Connective tissue cells, which play an important role in reparative changes, were observed.

Discussion

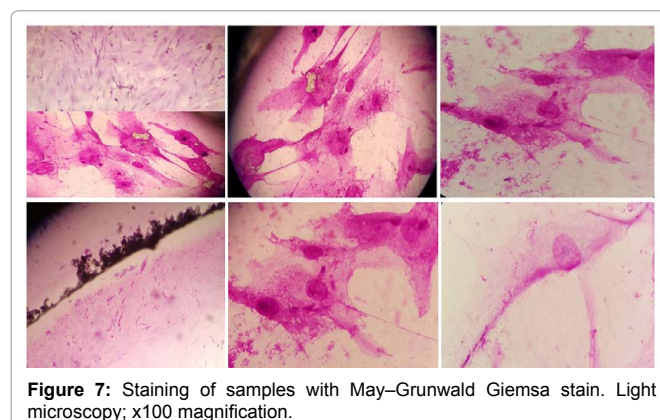
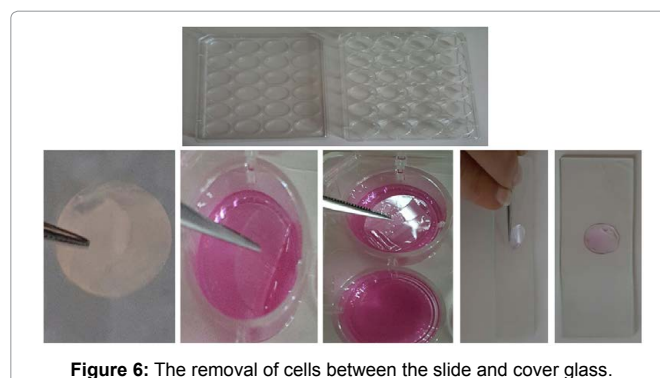
In parallel with advancing technology, an exponentially increasing number of studies are being performed in the cell culture

field. Orthopedic surgeons taking part in this research may create the background of their own studies, and they should strive to do so.

The present demand involves producing effective, living cells to allow proliferation in the culture environment and morphology. It is known that primary cultures bear the characteristics of the tissues in which they have been produced, and they generally have a heterogeneous structure [1-8].

Maran et al. embedded 2-methoxyestradiol into oligo (polyethylene glycol) fumarate-sodium methacrylate- and oligo (polyethylene glycol) fumarate-sodium methacrylate-poly(lactic-co-glycolic acid)-based hydrogels; 2-methoxyestradiol is known to kill OS commercial cells without affecting healthy osteoblasts [15]. Moreover, Ma et al. evaluated dox or PLK1shRNA (polylysine-polyethylenimine-loaded PLGA-PEG-PLGA triblock copolymer hydrogels by means of nuclear magnetic resonance spectroscopy [15]. Following physicochemical tests, the authors examined apoptosis and MTT cell viability, toxicity, and proliferation analyses in MG-63 and SaOS-2 commercial cell lines; they interpreted the PLK1 expression levels of OS cells using real time-polymerase chain reaction [16].

Liu et al. reported that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in cancer tissues without showing toxicity in normal cells [26]. However, they also reported that most OS cells are resistant to TRAIL. They reported that treatment methods are necessary that can eliminate TRAIL resistance in cancer treatment. They examined the combination with celecoxib, a non-steroidal anti-inflammatory drug, and showed that both drugs induce apoptosis in low doses. They examined this effect in MG-63 human OS cancer cell line cultures and reported that this effect occurred as a result of downregulation of cellular B-cell lymphoma (Bcl-2), Bcl-2-associated X protein, caspase-8, and caspase-3 .



Andronescu et al. studied composite materials involving a combination of collagen/hydroxyapatite; they loaded cisplatin onto the materials at doses of 6 and 10 μM near bones [27]. They examined the physicochemical features of the drug delivery system releasing cisplatin and the biological activities of the drug-release features. Moreover, they studied cytotoxicity and antiproliferative and anti-invasive activity in G292 OS cells. These researchers suggested that a collagen/hydroxyapatite composite loaded on cisplatin could be used as a local chemotherapeutic in future.

Duchi et al. stated that a therapeutic drug delivery system should be used by exploiting the feature whereby it hosts the tumor stroma of mesenchymal stem cells, which contain fluorescent core-shell poly(methyl methacrylate nanoparticles loaded with photosensitizer, which is called porphyrin [28]. They reported that these mesenchymal stem cells kill OS cells after photo-activation. In addition, Blattmann et al. sought to establish an orthotropic xenotransplanted mouse model using patient-derived tumor tissue for the treatment of OS, which is the most frequent pediatric malign bone tumor [17].

In the literature, studies have indicated that samples with heterogeneous cultures that are produced safely in laboratories are more appropriate than the use of samples comprising of monotype cells, such as cell lines [1,2,8,29,30]. As the nucleocytoplasmic rate may be much higher, gradient centrifugation is used to distinguish these cells from others. Specific markers used in the characterization of cells are cell surface receptors and cytoplasmic receptors or proteins. Both protein groups vary in terms of their affinity with many components, such as antibodies or signaling molecules. It is still possible to distinguish cells by signaling molecule that have an affinity to cell strains peculiar to marker or antibodies marked by fluorescence [8,31-33]. We used collagenase enzymes to isolate cells containing the most appropriate tumor cells.

The key question after the isolation step was which cell type should be fed with which type of CCMC. Another important point is that xenocultures and pharmaceuticals used as the cells and medium should be chosen from equipment that is tested against infectious agents in compliance with guidelines like those provided by the United States Food and Drug Administration. What is more, biochemical that will be used in the cell environment should be tested for endotoxins.

Cell reproduction occurs on sixth day. If the cells shapes have bright ranges and their center has a dark area with round shapes, it is a sign that the cells are healthy. If they are flatter and surrounded by fibroblast-type cells, it is a sign of the start of differentiation of the colonies. If not desired otherwise, it is important not to let cells become bigger and excerpted. Although these methods help us to distinguish the cells, the results should be verified by gene expression analyses [8,34,35].

Apart from the described methods, which are cellular based, some other analyses can be used. Mass spectrophotometry can be employed for the determination of polymerase chain reaction of the gene expression of cells or microarrays and proteomics profiles. In addition, this approach can be used for the measurement of specific protein interaction expression. Western blotting analyses can be used for protein measurements (e.g., collagen type) after sodium dodecyl sulfate polyacrylamide gel electrophoresis. In addition, next-generation techniques, such as chain analysis, which is array based,

can be applied to determine the gene expression or post-translational modifications [8,36-39]. Apart from these approaches, microRNA (miRNA) applications, in which mesenchymal stem cells are used, are still being investigated.

Hu et al. studied the effect of MIR-182 expression in OS. They examined MIR-182 levels using quantitative reverse transcription polymerase chain reaction method [40]. These researchers analyzed cell functions after transfection of miRNA inhibitors using cell counting kit-8, which allows sensitive colorimetric assays for the determination of cell viability in cell proliferation and the cytotoxicity assay, migration, and invasion assay methods. They verified miRNA-182 target genes using a bioinformatics algorithm. They reported that MIR-182 was lower than OS cells and tumor growth, with extreme expression and diminished migration and invasion. They emphasized that this inhibition was realized through T-lymphoma invasion and metastasis-inducing protein-1 (TIAM1). They also emphasized that MIR-182 expression induced TIAM1, and OS inhibited proliferation and invasion, which caused MIR-182 to behave as if it had been a tumor suspension gene [40].

Muff et al. emphasized the content of human sarcomas and the limitation of the use of tumors in preclinical research [41]. They offered the explant plant technique to project cell lines and deduced that it enables molecular analysis and drug testing.

Buddingh et al. examined mesenchymal stem cells of 9 cases with OS and 5 healthy cases for 649 days [42]. They found that binucleate cells increase in each excerpt. Moreover, they reported that hematopoietic cell-specific Lyn substrate 1 expression was four times lower than the cases with OS. However, they underlined that they did not find malign transformation in cultured samples obtained from healthy donor or OS cases [42].

Living mammals and cell lines have often been used in studies focusing on inhibiting or exterminating tumor activities. It is known that the sensitivities of human and animal tissues differ from each other, so the results may be misleading [1,2,7,8,12,43]. Using monotype cells, no complex coordination mechanisms concerning their micro-environments and their matrix structure inhibit interaction, which makes *in vitro* test results more controversial in terms of their compatibility with *in vivo* results. Furthermore, the biggest disadvantage of cell lines is that they do not have genotypic and phenotypic characteristics, as they are genetically modified cells [29,30,44]

The analyses in this present study were not carried out using cell lines; instead, they involved human primary explant OS cultures obtained from tumor tissues. Thus, the cells may have interacted with surrounding forms. Moreover, the culture process was carried out with consideration of possible allergenic reactions of the cases to drug and/or egg protein, because the tissues from allergenic cases would have been excluded from the study. If a protein=and/or peptide structure-derivative drug had been examined, the results may have been misleading, as would not have been possible to determine whether the damage and/or toxicity in cell culture resulted from drug or from idiosyncratic protein hypersensitivity peculiar to the case. For this reason, we consider that the data in our study show strong evidence. As the resection material obtained from the case belongs to a case who received chemotherapy, cultured cells were scanned as active fibroblasts which proliferated depending on necrosis and/or chemotherapy. After the required ethical approval is received, the culture steps should be repeated by including biopsy materials obtained from cases who did not receive chemotherapy.

We presented a detailed culture-preparation method in this study. We consider that this study can guide practitioners in terms of the preparation of primary human cell cultures.

Conclusion

After required approvals are received, if tumor tissues are properly placed into culture vessels, orthopedic surgeons can successfully use primary tumor cell cultures. If they produce appropriate and dependable cell cultures, cancer research studies may proceed on a firm footing. In addition, by means of primary cell culture not only for drug applications but also for adoptive.

Conflicts of Interest

The authors declare no conflicts of interest. All authors certify that neither they nor members of their immediate family have funding interests or commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that may pose a conflict of interest in connection with the submitted article.

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