Chapter

Application of Adipose-Derived Stem Cells in Treatment of Bone Tissue Defects

Stevo Najman, Jelena Najdanović and Vladimir Cvetković

Abstract

Despite excellent self-regeneration capacity of bone tissue, there are some large bone defects that cannot be healed spontaneously. Numerous literature data in the field of cell-based bone tissue engineering showed that adipose-derived stem cells (ADSCs) after isolation could be subsequently applied in a one-step approach for treatment of bone defect, without previous in vitro expansion and osteoinduction. However, standard approaches usually involve in vitro expansion and osteoinduction of ADSCs as an additional preparation step before its final application. Bioreactors are also used for the preparation of ADSC-based graft prior application. The commonly used approaches are reviewed, and their outcomes, advantages, disadvantages, as well as their potential for successful application in the treatment of bone defects are discussed. Difficulty in spontaneous healing of bone defects is very often due to poor vascularization. To overcome this problem, numerous methods in bone tissue engineering (BTE) were developed. We focused on freshly isolated stromal vascular fraction (SVF) cells and ADSCs in vitro induced into endothelial cells (ECs) as cells with vasculogenic capacity for the further application in bone defect treatment. We have reviewed orthotopic and ectopic models in BTE that include the application of SVFs or ADSCs in vitro induced into ECs, with special reference to co-cultivation.

Keywords: stromal vascular fraction, adipose-derived stem cells, endothelial cells, in vitro-induced differentiation, bone tissue engineering, vascularization, osteogenic process stromal vascular fraction, adipose-derived stem cells, endothelial cells, in vitro-induced differentiation, bone tissue engineering, vascularization, osteogenic process

1. Introduction

Structure of the bone tissue is very dynamic due to environmental influence but also because of many factors that act inside the body [1]. The bone can regenerate and repair itself, but large fractures and bone defects fail to heal and repair successfully. In addition to other adverse factors, this results in delayed unions, malunions, or nonunions. To aid bone healing and repair in such situations, build bone-deficient areas, or replace missing bone as well as in purposes of joint reconstruction, bone grafting is used [2–5].

Bone grafting is one of the most common options for the treatment of major bone defects, and the use of bone grafts is among the most common procedures

in orthopedic surgery and the second most frequent transplantation of tissue after blood. Over 2 million bone grafting procedures are performed annually in surgery [5, 6]. Bone graft material, alone or in combination with other materials, can perform bone healing function by having at least one of the features among osteogenicity, osteoinduction, and osteoconduction and therefore usually have one or more components—scaffold, as an osteoconductive matrix that supports bone growth, osteoinductive proteins and factors, and osteogenic cells [5, 7]. The main types of bone grafting materials are autografts, allografts, xenografts, synthetic and biological tissue engineering biomaterials, and combinations of these materials [2–6]. Bone grafts and graft substitutes may differ by material type, source, and origin and may also be categorized as osteogenic, osteoinductive, and osteoconductive agents [7]. The choice of bone graft depends on the condition of the bone tissue, defect size, surgical feasibility of the procedure, possible health complications, graft structure, its biological and mechanical characteristics, size and shape, cost, and ethical issues [5].

Each of the bone graft material and its substituents has its advantages and disadvantages, of which there is considerable agreement in surgical practice. Autografts are the best clinical solution among grafts because they have all the necessary properties to stimulate bone repair and regeneration—osteogenicity, osteoinductivity, and osteoconductivity—as they provide osteogenic cells, osteoinductive factors, and osteoconductive scaffold for bone growth. So far, autografts have proven superior in quality and time to bone healing. That is why in orthopedic practice, for the purpose of reconstruction of small defects and replacement of lost bone, autografts are the "gold standard" [3, 5–7]. Autografts can be of different types such as vascularized grafts, bone tissue, or bone marrow. They are usually obtained from the iliac crest, as well as from mandibular bone in dentistry, and by osteectomy and osteoplasty in various procedures. The vascularized fibula is used for the treatment of congenital bone damage, replacement of the bone segment after trauma, and in the case of a malignant tumor so that the periosteum and nutrient artery allow the graft to live and grow in the transplanted site [4, 8, 9]. The use of autografts often involves additional surgery and additional pain, and there is a frequent occurrence of morbidity at the donor site, which with limited availability is a limitation to their use. Morbidity, although low, is significant and ranges from minor to major complications, and there is a risk of injury to large blood vessels and visceral organs while taking the graft. The type of complications and their severity depends on the donor site as well as whether the graft material is taken at the same incision on the primary surgical site [2, 3, 5–8].

Allografts are much more available for orthopedic purposes. They have an osteoconductivity property, they can be well remodeled, but they are poorly osteoinductive, which can be present if they have an organic matrix. All of these properties depend on the size of the graft and the grafting site [3–6, 8]. Allografts are obtained from a bone bank containing cadaveric bones and can be found in various forms. Compared to autografts, allografts lack osteogenic properties and thus have an overall weaker bone regenerative potential, and their integration into the recipient bone over the long term may be insufficient. Allografts can induce immune response and rejection and requires caution because of the possibility of transmission of pathogens from the donor organism, and their disadvantages are also high costs and lack of availability of donors [3, 5–8]. To reduce some disadvantages and limitations by allografts, cells and proteins that can elicit an immune response can be removed, thereby also reducing the possibility of transmission of viral infection by donors. Usually, the sterilization and deactivation process of the proteins is performed before the use of the allograft, but then they lack osteoinductive factors, which in turn remain if the demineralized bone matrix is prepared [4, 8].

Xenografts are obtained from species other than humans, e.g., bovine bone, which is much used in dentistry. There are also nonbone xenograft materials such as sclera, collagen membranes, and coral-derived materials. Compared to autografts, xenografts as allografts only have osteoconductive and osteoinductive but lack osteogenic features. There is a risk of zoonotic transmission in xenografts treatment, and rejection is more likely and stronger than in the case of an allograft. Xenografts are cheaper, but the results of their application are inconsistent [4–6].

In addition to bone grafts (autograft, allograft, xenograft), newer bone substitutes are the ceramic types (calcium compounds: hydroxyapatite, tricalcium phosphate (TCP), calcium sulfate) and biological factors such as growth factors and others (bone morphogenetic proteins (BMPs), platelet-rich plasma (PRP), demineralized bone matrix (DBM)). Bone substitutes have been used for decades and have been defined as synthetic, inorganic, or organic, as well as biological origin materials, or a combination of those used to treat bone defect instead of bone [3, 4, 6]. Bone substitutes are especially used in traumatology and oncologic, spine, and prosthetic surgeries. Suitable bone substitutes should be biocompatible, not provoking an adverse inflammatory response, osteoinductive, osteoconductive, resorbable, easily molded into the bone defect, nonconductive, sterilizable, available, traceable in vivo, and at a reasonable cost [6]. The integration of bone substitutes over the long term may not be sufficient. Bone substitute materials of a synthetic nature such as calcium phosphate (CaP)-based biomaterials often behave osteoconductively only and can be remodeled. Osteoinductivity is possessed by biological factors such as bone matrix proteins or BMP-type growth factors that can be added to other bone substitutes [3, 8]. Ceramic-based bone graft substitutes include hydroxyapatite (HA), TCP, calcium sulfate, and bioglass used alone or in combination. Ceramics can be used in the form of granules, blocks, or moldable paste shape, and the occurrence of injectable cements was particularly significant because it enabled a mininvasive application [6, 7, 10]. Calcium phosphate-based bone substitutes have wide clinical use, since they are generally therapeutically effective, although they have poor mechanical properties, are less strong than bone tissue, and can be completely resorbed. The more advanced variants of HAs have biomimetic properties, since they include ions (carbonates, Mg, fluoride, Sr), so that natural HA is imitated [6]. Polymer-based bone substitutes can be degradable and nondegradable polymers and are applied alone, as co-polymers, or in combination with other materials [7, 10]. Various marine biomaterials are also used as bone substitutes, including chitosan, corals, and sponge skeleton [7]. Although biological factors generally influence good bone formation, the clinical application is not widespread due to high prices and possible adverse side effects [3]. Growth factors as bone substitutes, such as BMPs, transforming growth factor-beta (TGF-beta), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), can be natural and recombinant and can be used alone or in combination with other materials [7]. BMPs are bone growth factors that are widely used in spine surgery and for the treatment of tibial nonunion. The efficiency of some biological factors, as is the case with the PRP and DBM, is still an open question [6, 10].

The shortcomings in the outcomes of bone healing were the impetus to seek new types of grafts and bone substitutes. On these motives, a bone tissue engineering (BTE) as a new field began to develop in the last decades of the last century, integrating multiple disciplines such as cell biology, developmental and molecular biology, biomechanics, biomaterials science, immunology, and others. With the progressive introduction of innovations and new technologies, BTE has been offering more and more solutions to reduce the disadvantages of traditional bone grafts and improve the process of healing fractures and defects by achieving

better graft incorporation, osteoconductivity, osteoinductivity, and osteogenicity [5, 9–11]. Combining tissue scaffolds, growth factors, cells (especially stem cells), and gene therapies, along with three-dimensional printing and other new technological products, makes BTE a promising option. BTE has progressed over time, producing grafts with increasing ability to regenerate and repair bone [5–7, 9–12]. Cell-based treatment is emerging as a more promising approach in regenerative medicine. Cells (e.g., osteogenic or mesenchymal stem cells and others) are used to create new bone alone or are seeded onto a support matrix or scaffold to form bone tissue in vitro [6, 7, 9, 10, 13]. On these principles, engineered vascularized bone grafts can be created with some similarities to autografts [9, 10, 14]. Of particular interest in BTE is the application of mesenchymal stem cells because of their multipotency and the presence of osteogenic potential [6, 9, 10, 13, 15]. Cells can also be used as a vehicle for osteoinductive genes [6, 10]. Recent developments include ex vivo bioreactors capable at the very automated and controlled way to imitate in vivo environment producing bone with appropriate biomechanical properties before implantation [7, 9, 10, 12, 13]. To test the new features and products of BTE, as well as preclinical testing, many in vitro and in vivo methods and models have been developed with various advantages and disadvantages respectively [11, 16].

2. Sources of cells for tissue-engineered grafts

Nowadays, adult stem cells can be purified from different adult tissues and used in BTE as potential progenitors of osteogenic cells. It is reported that stem cells intended for BTE can be derived from many different tissues such as bone marrow, umbilical cord, dental pulp, as well as adipose tissue [17]. For instance, mesenchymal stem cells (MSCs) derived from bone marrow can be successfully differentiated into cells of various connective tissues [18] as well as in bone cells which give opportunity for its implementation in cell-based BTE [10, 19]. Despite its frequent use in BTE, bone marrow MSCs are reported to have some disadvantages, especially low cell quantity at isolation [20]. This is also an unfavorable characteristic for many other adult stem cells which imply in vitro cell expansion after their isolation in order to obtain sufficient quantity of cells which is mandatory for its further implementation in cell-based BTE. Morbidity and pain are also reported as accompanying side effects that are consequences of bone marrow MSC harvesting [20]. Nevertheless, MSCs are easier to isolate from accessible adipose tissue, which can be harvested with minimally harmful [21] and less painful methods. These multipotent cells derived from adipose tissue were reported by Zuk et al. [22] and suggested as a possible alternative to bone marrow MSCs [22]. Others also reported that stromal cells derived from adipose tissue rapidly proliferate and can be obtained in sufficient quantity, eliminating the need for in vitro expansion [23, 24] which is a tremendous advantage of adipose-derived stem cells (ADSCs) over other tissues that are potential sources of adult stem cells. The more recent study showed that bone morphogenic protein 2-transduced human adipose-derived MSCs had higher capacity for osteogenic differentiation than bone morphogenic protein 2-transduced MSCs from bone marrow [25]. With all this advantages, MSCs from adipose tissue are also reported to have similar potential to differentiate toward osteoblast and form bone as bone marrow MSCs [26]. Adipose tissue therefore represents a reliable source of adult stem cells intended for therapeutic purposes in BTE. In addition to this, in the past decade, we are witnessing an increase of interest for application of ADSCs in cell-based BTE.

2.1 Adipose-derived stem cells

The capacity to differentiate toward a broad spectrum of specialized cells such as bone cells, cartilage cells, muscle cells, endothelial cells, liver cells, neural cells, and others [17, 21, 27] makes ADSCs suitable generally for cell-based tissue engineering as well as for BTE. ADSCs represent a form of MSCs [28] that could be simply isolated from both subcutaneous and visceral adipose tissue that are usually sufficiently abundant in almost every individual. Nevertheless, it is reported that ADSCs derived from visceral fat have higher osteogenic potential than ADSCs from subcutaneous fat in rabbits [29] which should be taken into consideration when selecting the type of adipose tissue as starting source for ADSC isolation. Adipose tissue in humans could be obtained by liposuction or resection method that are both reported to yield similar quantity and good quality of MSCs [30]. More precisely, a direct source of ADSCs is stromal vascular fraction (SVF) which is obtained by enzymatic digestion of previously isolated fat tissue and its subsequent centrifugation [15]. There are well-described markers intended for ADSC characterization. Particularly, ADSCs are reported to have recognizable fibroblast-like, spindle-shaped morphology [22, 26], good longevity and plastic adhesion properties in in vitro condition [31], and capacity for rapid proliferation in cell culture [23, 32]. ADSCs also have characteristic expression pattern of CD cell surface markers. In general, there is an accordance in literature about the expression pattern of CD markers which should be used for ADSC characterization. For instance, Cai et al. [27] summarized that some characteristic CDs, particularly CD166, CD105, CD90, CD73, CD44, CD29, and CD13, have high expression in adipose-derived stem cells [27] which is in accordance with other reviews [33]. Also, according to Bajek et al. (2016) high expressions of CD73, CD44, CD105 and CD90 are indications mainly used for adipose-derived stem cell identification [34]. Therefore, before further steps in their application and analyses, it is very important to examine and determine expression of CDs and other markers like cell morphology in order to characterize ADSCs.

3. Bone substitutes and regulatory factors

As it is mentioned in the previous text, apart from cells, there are two more components in cell-based BTE, and those are bone substitute materials and regulatory factors. These three components together, combined in appropriate manner, makes specific construct which is an adequate alternative to bone grafts for the treatment of bone tissue defects. Bone substitutes are used as appropriate scaffolds for osteogenic cells. Chao et al. [12] described scaffold as logistic templates for guided formation of tissue [12]. Three-dimensional scaffolds that support osteogenic differentiation of the stem cells are seen as crucial components for in vitro engineering of bone construct which can be clinically usable [9]. Even more, to respond to special requirements at the defect site, engineering of the customized bone grafts was also proposed [35]. Bone substitute materials provide appropriate microenvironment for differentiation and proliferation of bone cells [36], and porosity, particle size, and material composition also play important roles [37]. At the first place, bone substitutes should be biocompatible which, among the other things, implies that they are non-toxic and non-genotoxic [38]. Biomaterials are also used to fill defects and compensate lost part of bone tissue at the defect site. Bone substitutes that belong to a group of bioactive biomaterials are capable for interaction with the biological environment and can provide conditions for cellular actions [39]

which is of benefit for bone regeneration. Nowadays, many different synthetic and natural bone substitutes are reported to be in use [10, 39, 40]. Particularly, materials based on hydroxyapatite and β-tricalcium phosphate are suitable for BTE [41]. In addition, materials based on bone mineral matrix are frequently used [42–45]. Also, regulatory factors are not less important components in BTE, and at the first place, they should induce and support osteogenic differentiation, adhesion, and proliferation of implanted cells. Because of its significant properties, here the focus will be on platelet-rich plasma as a source of regulatory factors for cell-based BTE. PRP is one of the well-known natural sources of different stimulative regulatory factors [46–48]. Last decade, PRPs are constantly drawing attention of many researchers in fields of regenerative biology as well as in BTE. That is reasonable because regulatory factors from PRP is reported to enhance adhesion, differentiation, and proliferation of the cells and also enhance angiogenesis [46, 49] which may support regeneration and reparation of bone tissue. In addition, it was reported that regulatory factors from PRP can enhance osteogenic process by inducing proliferation and differentiation of MSCs [50, 51]. Nevertheless, the question about adequate and stimulating concentration of platelets in prepared PRP is of great importance, and it is still topical. The opinions about adequate concentration of platelets in PRP intended for BTE are different. There are reports that higher platelet concentrations might have an inhibitory effect [52], and on the other hand, concentrations that are lower than the physiological level is reported to be useful for bone regeneration [53]. Finally, there is a recommendation that optimal concentration of platelets in PRP intended for bone treatment should be from four to eight folds higher than the normal physiological level of platelets in the blood [46]. Another advantage of activated PRP is that it can form fibrin fibers which can couple ADSCs with bone substitute material and improve retention of all construct components [54, 55] similar to reports where fibrin was used for this purpose [56, 57]. Finally, there is a growing interest about synergistic effects of ADSCs and PRP for bone regeneration. Many studies reported promising osteogenic potential of ADSCs and PRP combination [45, 54, 55, 58] and stimulative potential of PRP that improves osteogenesis in combination with cells [50, 59]. In addition, Fernandes and Yang [48] reviewed and summarized some recently published data which implies that adipose-derived stem cells obtained from human, mouse, rat and rabbit in combination with PRP in in vitro and/or in vivo conditions are related with outcomes which are of benefit for BTE [48].

4. Approaches in application of ADSCs in cell-based BTE

4.1 One-step versus multistep approaches

Numerous literature data in the past decade are related with extensive studies about regenerative potential of adipose-derived stem cells. It is well-known that ADSCs in combination with bone substitute materials and regulatory factors possess certain osteogenic potential that can initiate and boost osteogenic process both in orthotopic and ectopic conditions. Recent studies showed that good yield of ADSCs after isolation from adipose tissue provides opportunity for its subsequent application in one-step approach in cell-based bone tissue engineering without previous in vitro expansion and differentiation. Nevertheless, standard approach in cell-based BTE usually involves in vitro pretreatment of ADSCs as an additional step before its final application in order to induce differentiation of ADSCs into osteogenic cells. Literature data showed that both approaches are promising for implementation in treatment of bone tissue defects. Our experience about

osteogenic capacity of differently prepared ADSCs is mostly in accordance with other related studies. Based on our previously performed experimental studies and published results, these two approaches have quite different outcomes, and each approach has its advantages and potential to be successfully applied in treatment of bone tissue defects. Also, there is a growing number of researches that employed bioreactors for bone graft engineering.

There are well-described methods for ADSC preparation prior their application in BTE. Without purification, in vitro expansion, and osteoinduction, ADSCs contained in freshly isolated adipose-derived SVF could be prepared and applied in just one step which is described as intraoperative approach [56, 57, 60] or one-step procedure [61]. Intraoperative approach implies construct assembling during surgical procedure [13] by combining cells, bone substitutes, and regulatory factors together in one construct. One of the earlier reports by Aslan et al. [62] presented usage of noncultured human MSCs isolated from bone marrow [62]. More recently published articles demonstrated intraoperative application of freshly isolated SVF cells from human adipose tissue [56, 57] and freshly isolated adipose-derived SVF cells from mice epididymal adipose tissue in ectopic bone-forming model [55]. One-step surgical procedure is also used in oral and maxillofacial surgery for maxillary sinus floor elevation [37, 63]. All these studies suggest that previous in vitro pretreatment of ADSCs and their separation from SVF population prior their implementation in BTE is not necessary. Good yield of ADSCs enables bypassing of in vitro expansion step which goes in favor of the one-step concept in BTE. In addition, the autotransplantation of freshly isolated ADSCs for the treatment of bone tissue defects also enables avoiding problems with immune response [60] that might occur in allotransplantations and xenotransplantations. Adipose-derived SVF is reported to have great capacity for regeneration processes thanks to its heterogeneity [28] because it is composed of different cell types and growth factors [15], especially with cells that have osteogenic and angiogenic potential [37] which is of great importance for treatment of bone defects (**Figure 1**).

Also, it is reported that the entire procedure can be finished in a few hours [13, 56, 64] which reduces wait time for surgery [60] and is one of the major advantages of this approach. In addition, Jurgens et al. [61] showed that ADSCs can adhere promptly onto materials collagen type I/III and poly(L-lactide-co-caprolactone); particularly it is reported that nearly 10 minutes is sufficient time for ADSC adhesion [61] that significantly reduces time needed for construct preparation.

On the other hand, there are many well-described approaches which involve several steps for in vitro expansion and osteogenic differentiation of ADSCs before its final application [26, 45, 65]. In these approaches, by in vitro cultivation, ADSCs are additionally expanded and purified from heterogenous SVF [32] that are directly derived from adipose tissue (**Figure 1**). After that step, purified ADSCs are subjected to in vitro induction in osteogenic media to differentiate toward osteogenic cells before further application which follows in the next step. The characteristic components of osteogenic medium are dexamethasone, ascorbic acid, and β-glycerophosphate [31, 33, 66] which are frequently used for ADSC osteoinduction [67]. There are different data about the duration of in vitro osteogenic induction needed for ADSC differentiation toward osteogenic cells, but literature data mostly referred to 2 weeks [22, 33, 66, 68, 69] or between 2 and 3 weeks [21] (**Figure 2**). It was summarized that during osteogenic differentiation, ADSCs start expression of lineage-specific genes for osteogenesis such as osteocalcin, transcription factor osterix, transcription factor Runx2, bone sialoprotein, alkaline phosphatase, and others [32, 40] which might be detected by gene expression analysis [61] as confirmation of successful osteogenic differentiation of ADSCs. For instance,

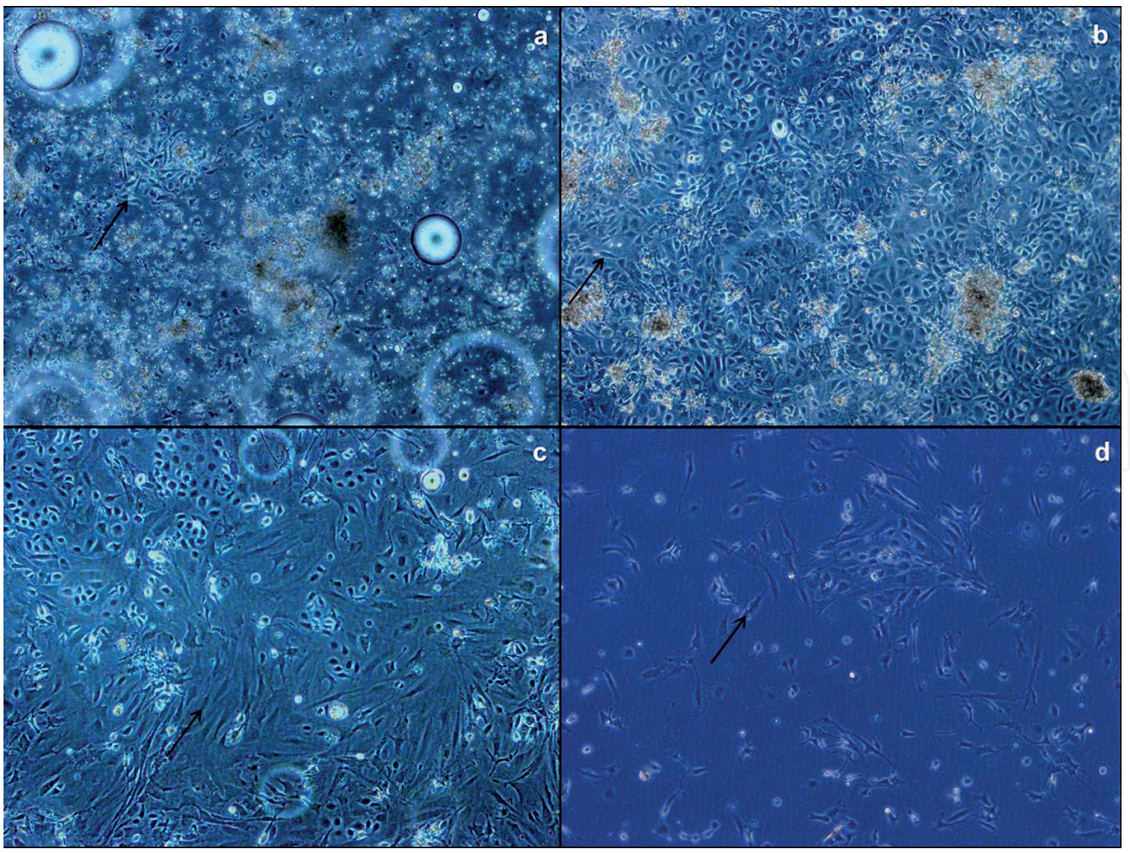

Figure 1.
Expansion and purification of ADSCs from adipose-derived SVF through in vitro cultivation. (a) Adipose-derived SVF 24 h after isolation; (b) adipose-derived SVF 72 h after isolation; (c) adipose-derived SVF 7 days after isolation; and (d) ADSCs the first day after the first passage. Black arrows show ADSCs. Magnification 100×.

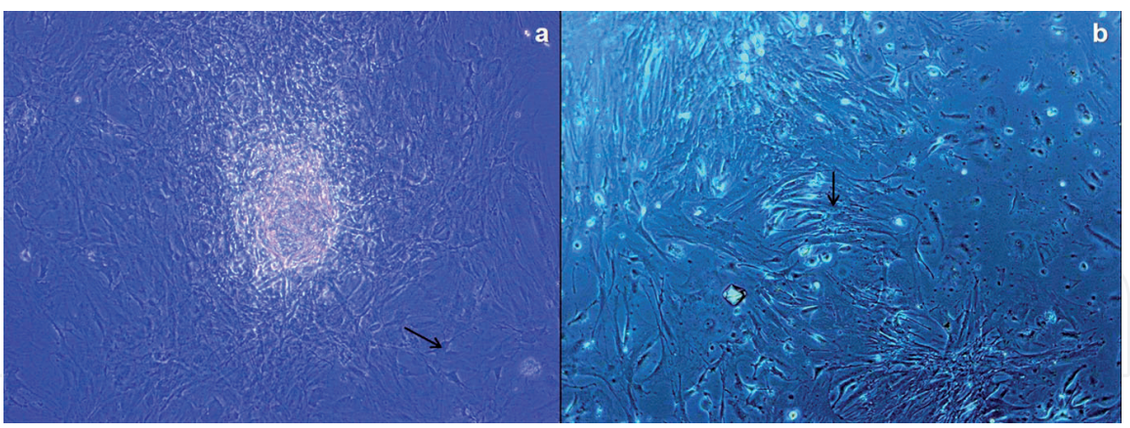

Figure 2.
In vitro osteoinduction of ADSCs. (a) ADSCs 3 days after cultivation in osteogenic media; (b) ADSCs 2 weeks after cultivation in osteogenic media. Black arrows show ADSCs. Magnification 100×.

Cvetković et al. [45] recently published that bone-related genes osteocalcin, transcription factor osterix, alkaline phosphatase, and collagen I alpha1 chain had the highest expression in in vitro osteoinduced ADSCs at 15th day of osteoinduction [45]. Also, it is reported that changes in cell morphology during osteogenic induction to a more cuboidal shape could be observed in ADSC culture [69]. The presence of mineralization signs and proliferation of the ADSCs are also reported as markers of osteogenic differentiation during in vitro osteoinduction [65, 69, 70]. Particularly, after in vitro osteogenic differentiation, mineralization of the cell matrix could be evaluated using Von Kossa and alizarin red staining [31, 66]. In the next step, when osteoinduction

is confirmed, osteoinduced ADSCs are seeded on bone substitute material and supplemented with regulatory factors prior using in BTE. There are examples where prepared constructs, after ADSCs seeding on biomaterial (i.e. bone substitute material), are subjected to in vitro osteoinduction before implantation [26] which requires additional time for construct preparation and delays final application. Overall, in vitro osteoinduction is proven to be an effective method for preparation of the ADSCs, and that is confirmed in many different studies [26, 45, 65] which makes this method suitable for BTE. But methods that consider at least two steps usually few weeks for performing and additional material for preparation of ADSCs are time- and money-consuming and more complicated to perform than the one-step method.

According to literature data, both in vitro osteoinduced ADSCs and untreated freshly isolated ADSCs are capable to induce osteogenesis to some level in orthotopic as well as in ectopic conditions. However, there are differences in their capacity to initiate and maintain osteogenic process. By comparing our two recently published studies, it could be concluded that ADSCs prepared and utilized in different manners induced different expression patterns of analyzed osteogenic markers in ectopic implants [45, 55]. In one of the studies, it was shown that untreated ADSCs contained in freshly isolated SVF are capable to quickly initiate osteogenic process, but between the 4th and 8th week of implantation, decreasing bone-related gene expression was detected [55]. On the other hand, Cvetković et al. [45] reported that in vitro osteoinduced ADSCs cause steady osteogenesis with peak at the 8th week [45]. In addition, there is a study which confirmed that osteogenically differentiated human ADSCs induced forming of bone tissue after 8 weeks in ectopic condition [26]; thus the application of osteoinduced ADSCs seems to have a favorable effect on osteogenesis. In other words, all these findings indicate that freshly isolated untreated ADSCs cannot maintain osteogenesis in ectopic condition to that extent as in vitro osteoinduced ADSCs can do. Previous osteoinduction triggered differentiation of ADSCs toward osteogenic cells which had sufficient potential to start and maintain osteogenesis for a longer period [45]. It was concluded that one of the reasons why untreated ADSCs within SVF failed to maintain osteogenic process for a longer period may be because of the lack of osteogenic factors in the ectopic environment which was used as model in this study [55]. Bone tissue normally had factors such as cytokines, mechanotransduction, and closeness of osteoprogenitor cells that are reported to act stimulative in bone formation, but they are reduced in ectopic bone-forming models [71]. Therefore, these factors are characteristic only for orthotopic models which allow evaluation of osteogenic potential of examined engineered construct in real natural milieu of the bone tissue. For that purpose the frequently used orthotopic models in BTE are critical-sized defects in calvaria bone [23] and long bones [44, 72, 73] where large rodents and other mammals are suitable. In addition, it was reported that the presence of sufficient doses of osteogenic factors are needed to support osteogenic differentiation of implanted cells and bone formation in ectopic conditions [56, 64].

Deficiency of osteogenic factors in ectopic models may be bypassed to some extent by addition of stimulating factors to ADSCs such as activated PRP. The addition of activated PRP can make microenvironmental conditions similar to the natural ones that occur during bone trauma. But it is known that platelets release a major portion of regulatory factors immediately after injury [74], which might be sufficient to stimulate ADSCs immediately after its implantation but definitely not for a longer period. The manner and applied doses of PRP obviously was enough to support development and maintenance of osteogenesis guided by previously in vitro osteoinduced ADSCs [45] and was not sufficient to support maintenance of osteogenic process guided by untreated ADSCs within SVF [55]. Regarding the short period of efficient action of PRP, Fernandes and Yang [48] reported that

there is a need for carrier that would deliver PRP in the manner that it can act more efficiently and release growth factors sustainably [48]. This might be the key for enhancing osteogenesis guided by freshly isolated and untreated ADSCs within SVF. Despite disadvantages of ectopic models, there are many literature data where ectopic subcutaneous implantations were conducted [42, 43, 71, 75, 76] because they allow examining the real potential of the implanted cells [64] alone and without the impact of the factors that are normally present in bone tissue. Therefore, selection of adequate animal model should depend on experimental goal, and finally it is important for interpretation and extrapolation of obtained results [1]. Nevertheless, osteogenic capacity of previously discussed combinations of differently prepared ADSCs should be evaluated in orthotopic bone-forming models because they are closer to real situations where treatment of bone defect is needed. That is especially of great importance for intraoperative (one-step) method because it is still unclear if this method ready for clinical implementation. In addition, recently a safe and feasible one-step surgical procedure for maxillary sinus floor elevation with implants consisting of calcium phosphate and freshly isolated SVF was demonstrated [63].

Preparation and utilization of freshly isolated and untreated ADSCs at the first place provides tremendous acceleration of procedure performance which is very important especially in everyday clinical practice where intraoperative method is desirable. Despite much longer period which is needed for ADSC preparation and utilization, there is no doubt that osteoinduction is a reliable method for the purposes of BTE. Overall, according to studies published so far [45, 49] that are mostly in accordance with other related studies, it could be concluded that untreated ADSCs contained in freshly isolated SVF have different potential from in vitro osteoinduced ADSCs to start and maintain osteogenic process which leads to quite different outcomes, at least in ectopic condition. Surely, each of presented approaches has its own advantages and potential to be successfully applied in treatment of bone tissue defects. We believe that both approaches could be successfully utilized in the treatment of bone tissue defects, but additional research should be conducted especially in orthotopic models in order to determine required doses of osteogenic factors needed to support osteogenic process.

4.2 ADSCs-based grafts prepared in bioreactors

We previously discuss some methods for ex vivo engineering of the grafts in in vitro static condition using cells and bone substitutes as scaffold. The advanced method for graft engineering involves the use of bioreactors. Bioreactors are defined as devices for precise monitoring and controlling of conditions which are necessary for biological and biochemical processes [77]. The controlling and monitoring of the conditions are in favor of minimalizing variability of graft production as well as standardization of the process [13] for graft engineering. Amini et al. [10] reviewed several types of bioreactors which include perfusion bioreactors, rotating bioreactors, and spinner flask bioreactors. Perfusion-based bioreactors, which are marked as mostly used, significantly stimulate osteogenic cells due to fluid flow [10]. Thus, the dynamic conditions, which bioreactors provide, are more similar to in vivo conditions which allow proliferation and differentiation of seeded cells of three-dimensional scaffolds in a much appropriate way than static in vitro conditions. There is literature evidence where human ADSCs were successfully used for engineering bone grafts in stirrer flask bioreactors [78] as well as viable bone tissue construct in perfusion bioreactor [79]. However, the engineering of the ready-to-use bone grafts using bioreactors could last for weeks, and it must be performed in several steps. Also, bioreactor devices for BTE purposes have high prizes, and

the whole procedure is not economical because it requires a higher consumption of medium and other materials which reduces accessibility of this method.

5. Solving vascularization problems in bone tissue engineering by SVF and ADSCs in vitro induced into endothelial cells

5.1 The problem of insufficient vascularization in bone tissue engineering

Bone tissue is a self-renewable tissue with an excellent regeneration capacity [80]. Some bone fractures, tumors, or bone loss can cause such large bone deficiencies that cannot be healed spontaneously [80], since their size goes beyond bone self-renewing capacity [81]. In those cases, classical surgical procedures or bone tissue engineering strategies must be applied [82]. The main problem that has not yet been completely overcome is insufficient vascularization in critical-sized bone defects. Therefore, a perfect bone substitute must have excellent angiogenic features [83].

Until now, various BTE methods for resolving insufficient vascularization were performed: changing the architecture and interconnectivity of pores of the applied biomaterial [84], co-cultivation of different types of cells [85], using mechanical stimulation [86], and adding one [87] or a couple of growth factors in the implants at the same time [88]. The implants were placed into highly vascularized areas or, before the implantation procedure, seeded with cells that secrete chemoattractants for attraction of the host's cells and blood vessels with small diameter [89]. Also, microvascular fragments of adipose tissue were incorporated [90], and tissue flap procedures [91] and scaffolds made of nano- or microfibers were applied [92].

One of the possible methods to facilitate anastomosing between the bioengineered vascular structures with the ones from the surrounding tissue is the construction of prevascularized scaffolds that contain endothelial cells (ECs) [93]. ECs constitute a continuous monolayer among interstitial fluid and blood and have crucial importance in vascularization and in controlling the function of blood vessels [94]. By producing, metabolizing, and releasing numerous humoral and hormonal agents, these cells create an active antithrombotic surface in order to ease transit of plasma and cell components through the blood vessels [94]. Nevertheless, not only that ECs are important for successful developing of the functional vascular system, other cells that constitute blood vessel wall, smooth muscle cells, and perycites are also of great importance [9]. Mural precursor cells participate in vascular remodeling and contribute to better vascularization in cell cultures which is the reason why these cells are desirable as one of the cell lines in co-culture [95, 96].

An important question regarding the use of ECs, not only in BTE but in tissue engineering generally, is "which type of ECs should be used?" [97]. Sources of autologous ECs are limited [98], and mature ECs have limited proliferative capacity [99, 100]. In addition, the procedure of isolation is invasive, it is hard to collect a plentiful number of cells, and there is a possibility of contamination, infection, and change of phenotype and function of ECs during in vitro cultivation [94, 99, 101]. Also, ECs isolated from different organs manifest different phenotypes in vivo, which mean that EC types for the application in tissue engineering should not be selected randomly [102].

Bearing all this in mind, alternative methods have been developed in order to obtain more stable sources of ECs. These methods include in vitro induction of MSCs into ECs. An easily accessible tissue [103] that attracts a great attention in the last few decades is adipose tissue. Primarily cell components of adipose tissue are cells filled with lipids—adipocytes. Besides adipocytes, stromal vascular fraction of adipose tissue consists of microvascular ECs, pericytes, fibroblasts, macrophages,

leukocytes, pre-adipocytes, mastocytes, and adipose-derived mesenchymal stem [104, 105]. SVF with heterogeneous cell populations can be obtained upon simple enzyme-based adipose tissue isolation procedure that includes enzymatic digestion, filtration, and centrifugation [76, 106–109]. After this procedure, SVF can be used as a source of ADSCs directly [108] or after expansion in cell culture through few passages [104] and subjecting to differentiation toward certain cell line [66, 76, 105, 109–111].

ADSCs secrete numerous angiogenesis-related mediators including vascular endothelial growth factor (VEGF), bone morphogenic proteins, placental growth factor (PGF), angiopoietin-1, hepatocyte growth factor (HGF), transforming growth factor-β, and fibroblast growth factor 2 (FGF-2) [112]. Secretion of these angiogenic factors makes ADSCs convenient for regenerative cell therapy [106]. Among the abovementioned growth factors, VEGF and BMP2 are considered to be the main factors during bone regeneration, VEGF on the vascular and BMP2 on the osteogenic side [113]. Besides soluble growth factors, ADSCs release plasma membrane-derived vesicles (MVs) that can contain some pro-angiogenic and osteogenic molecules [114] with an influence on adjacent cells. In addition, growth factors and other molecules contained within MVs (cytokines, RNAs, microRNAs) can be transported to some more distant target cells all over the body. By taking up the content of MVs, target cells use these molecules and perform certain biological activity including angiogenesis of target cells which is of crucial importance in bone tissue regeneration [115].

In order to apply SVF as a source of cells with vasculogenic capacity in bone tissue engineering, several methods have been described. These methods include application of SVFs immediately after isolation from adipose tissue as well as after in vitro induction of endothelial differentiation.

5.2 Potential of stromal vascular fraction as a source of cells with vasculogenic capacity for application in bone tissue engineering

Freshly isolated SVF contains both endothelial and skeletal progenitor cells [116]. This property was widely used to construct osteogenic and vasculogenic grafts. It has been found that three-dimensional (3D) cultures of ECs and osteoblasts (OBs) as well as osteogenic-vasculogenic constructs could be achieved by using perfusion-based bioreactor system of single cell source human SVFs in ceramic scaffolds [116]. Namely, SVFs were seeded on 3D porous ceramic scaffolds and cultivated during 5 days using bioreactor system. Eight weeks after implantation of resulting scaffolds in nude mice, formation of functional vascular network that was connected with the host's vasculature as well as ectopic bone formation was observed. Nude mice model was also used by Todorov and his colleagues [117]. This team isolated SVFs from human abdominal lipoaspirates and obtained hypertrophic cartilage (HC) pellets from bone marrow-derived stromal cells. Devitalized HC was embedded in fibrin gel and implanted with and without SVFs ectopically and in calvarial defects. Twelve weeks after implantations, vascularization and bone formation of grafts enriched with SVFs were enhanced in ectopic, subcutaneous, and in orthotopic experimental model.

The use of human SVF-derived vascular progenitor cells can speed up the engraftment of critical-sized osteogenic constructs which improves in vivo formation of the bone tissue [118]. Human SVFs have been isolated, seeded, and cultured into hydroxyapatite scaffolds. Perfusion bioreactor system was used in order to preserve the cells of $CD34^+/CD31^+$ endothelial lineage from the SVF. As a result of in vitro cultivation, 5 days after seeding, endothelial and mesenchymal progenitors from SVF constituted capillary networks that were able to anastomose with the host vasculature 7 days after implantation on an ectopic nude rat model [118].

With an aim to simulate an intraoperative procedure on an ectopic bone forming model, autologous SVFs isolated from epididymal adipose tissue of Balb/c mice and platelet-rich plasma were added to the bone mineral matrix (BMM) [108]. BMM served as a carrier for cells and growth factors. The constructs were implanted subcutaneously, and osteogenic capacity of this combination was examined. Eight weeks after implantations, these implants had significantly higher percentage of infiltrated tissue and percentage of vascularization than the control (BMM-only implants). According to osteogenesis-related gene expression analysis, implants with SVFs induced rapid onset of osteogenesis process. One of the reasons for such results probably lies in the fact that freshly isolated SVFs had upregulated expression of endothelial- and osteogenic-related genes which was proved by using real-time PCR analysis.

Non-induced ADSCs cultivated in vitro up to the third passage were mixed with allogeneic PRP taken from three healthy Wistar rats [119]. ADSCs-PRP constructs were implanted into the jaws of rats that had bisphosphonate-related osteonecrosis of the jaw. Eight weeks after the treatment, the incidence of osteonecrosis was lower, while the degree of bone turnover and number of osteoclasts were higher than the experimental groups that were not treated with ADSCs [119]. These data could be explained by the fact that PRP release numerous growth factors that can fasten both in vitro and in vivo ADSC differentiation without previous addition of any other factors inductive toward certain cell line [109]. One of the possible mechanisms of synergistic effect of ADSCs and PRP could be ascribed to the release of platelet-derived growth factor from PRP. PDGF has an impact on selectable expansion and recruitment of non-induced MSCs, as well as on proliferation and migration of progenitors of blood vessel wall cells [120]. It also triggers differentiation of MSCs into blood vessel cells [107] and enhances release of extracellular vesicles (EVs)—MVs and exosomes from ADSCs which further influence both in vitro and in vivo angiogenic process [121].

On an ectopic model, Man and associates [109] pointed out to the effect of ADSCs that is similar to the one shown in the abovementioned orthotopic model. ADSCs isolated from inguinal rabbit fat pads and mixed with PRP were loaded onto alginate microspheres. Well-developed blood vessel network and good bone mineralization were observed 3 months after subcutaneous implantations. Opposite results were obtained in another research where non-induced ADSCs were seeded on poly (D, L-lactide) scaffolds and implanted in the large rat palatal bone defect [122]. Bone formation did not occur even 12 weeks after implantations, and the defects were filled with dense fibrous tissue. The differences between the outcomes of the different studies could be attributed to the fact that adipose tissue was taken from the different localizations of the donors' body. Also, the implant preparation was done in a different manner, and different models were chosen for the implantation procedure.

Adipose tissue can also be used as a source of microvascular fragments (MVF) that can be further applied as vascularization units [123]. MVF isolated from CD-1 mice were incorporated into thermoresponsive hydrogel (TRH), cultivated, and used for filling the osteotomy gaps in the femurs of CD-1 mice. Bone healing was assessed 14 and 35 days after induction of osteotomy, while non-incorporated MVF as well as no material group served as control groups. It was found that TRH is a suitable carrier for MVF since vascularization in MVF-loaded TRH was improved in comparison to the control groups. In contrast to this finding, bone formation in this group was impaired, probably due to low levels of VEGF expression during the early stages of bone healing.

Bone substitute biomaterials based on calcium phosphate, including hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), and HA/β-TCP combination, are

often used in BTE due to their good biocompatibility and the absence of toxicity of their chemical compounds [124]. These materials also have such 3D features that allow immediate colonization by MSCs and extensive revascularization [125]. For those reasons, Farré-Guasch and his associates used ADSCs-containing SVF seeded on calcium-based biomaterials to treat the patients subjected to maxillary sinus floor elevation (MSFE)—a surgical procedure that in some patients must precede dental implant placement [126]. Autologous SVFs taken from patients were seeded on two types of carriers for two different groups of patients: group 1 had SVFs seeded on β-tricalcium phosphate, and group 2 had SVFs seeded on biphasic calcium phosphate, while the control group had only ceramics, without cells. Histomorphometrical analysis and immunohistochemical staining for blood vessel markers such as CD34 and alpha-smooth muscle actin revealed higher number of blood vessels and immunoexpression of blood vessel markers in both experimental than a control group. These results point out pro-angiogenic influence of SVF.

One of the recently published papers regarding pre-vascularization of various engineered tissues compares the use of ADSCs as a potential source of cells with vasculogenic capacity in combination with different types of gel-based scaffolds [127]. ADSCs were isolated from human fat tissue, cultivated, and, after second passage, molded in fibrin as well as agarose-collagen gels. After 14 days of incubation have passed, the gels were analyzed by two-photon laser scanning microscopy. Vascularization was achieved in both types of gels which were detected as branched networks of tubular vascular structures in both hydrogels. Nevertheless, volume, area, and length of vascular structures supported by ADSCs in agarose-collagen hydrogels were comparable to human dermal fibroblast control.

5.3 Potential of adipose-derived stem cells in vitro induced into ECs for application in bone tissue engineering

5.3.1 Orthotopic model

In order to increase vascularization, ADSCs induced into ECs were applied in BTE by using several models, among which orthotopic model is one of the most commonly used. Rat ADSCs in vitro induced into ECs during 8 days was used for the construction of allografts [128]. The cells were combined with sterilized and decellularized banked allografts made of calvaria from earlier sacrificed Lewis rats. Allografts were implanted into critical-sized calvarial defects in rats, and 8 weeks after the implantation, blood vessel density was increased. As a consequence, bone volume was also increased. In parallel, two other types of allografts were constructed—allografts seeded with ADSCs induced into OBs and allografts seeded with the combination of ADSCs induced into ECs and ADSCs induced into OBs. These implants had weaker vascularization and lower bone volume 8 weeks after the implantation than the ECs-only allografts.

ADSCs in vitro induced into ECs were also seeded onto poly(D, L-lactide) scaffolds and implanted into critical-sized calvarial defects of Lewis rats [110]. Scaffolds prevascularized in this manner did not caused an increase in bone formation by itself, but according to the conclusion of this team, they could possibly be used as a source of cells for accomplishing better vascularization and function of the existing OBs. On the other hand, the constructs that were constructed out of undifferentiated ADSCs or ADSCs induced into OBs had statistically greater bone volume than the implants containing ADSCs induced into ECs.

In another study, also performed on critical-sized calvarial defect model of Lewis rats, hydroxyapatite/poly(lactide-co-glycolide) [HA-PLG] was used as a biomaterial carrier, while adipose tissue was extracted from inguinal fat pads [129].

ADSCs were induced into ECs as well as into OBs. Vascularization and osteogenic process were evaluated in the following groups: (I) HA-PLG scaffolds without cells, (II) HA-PLG scaffolds seeded with non-induced ADSCs, (III) HA-PLG scaffolds seeded with ADSCs induced into ECs, and (IV) HA-PLG scaffolds seeded with ADSCs induced into OBs. The highest bone mineral density, bone regeneration, and vascular density in regenerated bone were found in group IV, although none of the found differences had statistical significance ($P > 0.05$).

In order to repair critical-sized bone defects in rat femur, ADSCs induced into ECs were used for prevascularization of the modified hierarchical mesoporous bioactive glass (MBG) scaffold with an enhanced compressive strength and then combined with ADSCs subjected to osteogenic differentiation [108]. Prevascularized MBG carrying ADSCs induced into OBs had more advanced angiogenesis both on the surface and in the interior than the non-vascularized MBG carrying ADSCs induced into OBs and MBG scaffolds that were not seeded with cells. Moreover, the group with prevascularized MBG scaffolds had the highest mineral deposition rate postoperatively. These results indicate that time-phase sequential utilization of ADSCs on MBG scaffolds is a good strategy for reparation of massive bone defects.

5.3.2 Ectopic model

Ectopic models are important in bone tissue engineering since they provide reducing external influences and side effects, thus concentrating on intrinsic potential of the applied implant components [53] and their interactions [42]. With an aim to overcome the problem of inadequate blood vessels development and consequent inability of bone tissue regeneration, the influence of ADSCs in vitro induced into ECs on vascularization and osteogenic process in ectopic osteogenic implants was examined [109]. The implants composed of ADSCs, BMM, and PRP and the ones composed of BMM and PRP were subcutaneously implanted into BALB/c mice. Endothelial-related gene expression, high percentage of vascularization, and VEGFR-2 immunoexpression show that implants enriched with ECs have increased vascularization compared to the cell-free implants. This was followed by more pronounced signs of osteogenic process in ADSCs-BMM-PRP implants than in BMM-PRP implants. By examining endothelial-related gene expression, vascular cell adhesion molecule-1 (VCAM-1) and osteopontin immunoexpression, it was also shown that the composition of implants based on biological triad (ADSCs induced into ECs, PRP and BMM) is more favorable for improving vascularization in the ectopic bone-forming model than in the BMM-only implants [76].

Uninduced ADSCs cultivated up to the 12th day after the third passage, combined with PRP and BMM and subcutaneously implanted into BALB/c mice, also have vasculogenic potential [109]. However, their vasculogenic potential is lower than that of ADSCs in vitro induced into osteogenic cells and implanted in combination with BMM and PRP. Specifically, relative gene expression analysis of endothelial gene markers *Vwf*, *Egr1*, *Flt1*, and *Vcam1* was significantly higher *($p < 0.05$)* in the group that contained osteoinduced ADSCs than in the group with uninduced ADSCs for each single gene and with the exception of *Vwf* at 1 and 4 weeks after implantations, at each single observation point.

Endothelial differentiated ADSCs and osteogenic differentiated ADSCs can be simultaneously applied for the construction of ectopic osteogenic implants. A method of in vitro prevascularization applied by Zhang and his team included construction of double cell sheets (DCS) out of rabbit ADSCs previously in vitro induced into ECs and into osteogenic cells [113]. DCS were combined with coral hydroxyapatite (CHA) in four different manners. Twelve weeks after ectopic

implantation into nude mice, a group that contained CHA covered with DCS such that endothelial cell sheet was inside and osteogenic cell sheet outside exhibited the most favorable results regarding vascularization and bone maturation of the graft.

5.3.3 Co-cultivation

Co-cultivation of ECs with other cell types is one of the approaches for resolving the problem of inadequate vascularization in BTE [130]. ECs were co-cultivated with different types of cells before the implantation procedure, but the most important for BTE is co-cultivation of ECs and OBs since numerous interactions between these two cell types exist during normal bone regeneration process [131, 132]. One of the mechanisms of those interactions was found by Kaigler and associates [133]. They have shown that in vitro, ECs improve osteogenic capacity of bone mesenchymal stem cells (BMSCs) during co-cultivation, in direct contact or near each other. In part, this role of ECs could be attributed to release of BMP-2. In vivo, transplanted ECs enhanced capability of transplanted BMSCs to form the bones.

The positive effect of combination of ECs and OBs was estimated in ectopically implanted HA/bTCP scaffolds. The 3D porous ceramic scaffolds were seeded with in vitro co-cultivated ADSCs induced into OBs, ADSCs induced into ECs, and $CD14^+$ osteoclast progenitors derived from human peripheral blood osteoclasts [134]. This three-dimensional organotypic culture model based on human cells was cultivated during 21 days in the perfusion bioreactor system. After cultivation, the system was implanted subcutaneously into dorsal pockets of nude mice. Eight weeks after implantation, blood vessels and bone-like tissue were formed.

In another study, co-cultivation of ADSCs induced into ECs and ADSCs induced into OBs did not have such positive effect on vascularization and osteogenic process [130]. Co-cultivation increased proliferation of this two cell types in vitro in comparison with monocultures or undifferentiated ADSCs. Nevertheless, co-cultures in a ratio 1:1 = OBs:ECs seeded on polylactic acid gas-plasma-treated scaffolds have not induced increased vascularization and signs of osteogenic process compared to the implants constituted out of non-induced ADSCs and polymer scaffolds. Unlike the co-culture group, ECs seeded on polymer scaffolds have increased vascularization, and OBs seeded on polymer scaffolds have improved osteogenesis more than a group with undifferentiated ADSCs [130].

Unlike the results of the above discussed study, ADSCs in vitro induced into OBs and ADSCs in vitro induced into ECs and seeded into self-assembling peptide RADA16-I scaffolds as co-cultures (1:1) had better osteogeneration and vascularization than the scaffolds seeded with monocultures of this type of cells or non-induced ADSCs [135]. Before seeding the cells into RADA16-I scaffolds, it has been shown that the best interaction of ADSCs induced into OBs and ADSCs induced into ECs in co-cultures was achieved when the ratio of the cells was 1:1.

The results obtained by some of the abovementioned research teams, which were worse than expected regarding a group of implants that contained previously co-cultured ECs and OBs, could lie in the ratio of the applied cell types. It has been confirmed that application of too many ECs decreases graft neovascularization probably due to increase in metabolic load and enhanced competition for nutrients [136]. The other reason for those differences can be the differences in the applied biomaterials used for the implant construction [130].

5.3.4 Arteriovenous vascular bundle

Formation of arteriovenous vascular bundle (AVB) belongs to in vivo prevascularization approaches. Incorporation of AVB into scaffolds represents a model

of scaffold that have artery and vein inserted into its central part. In this way, stem cells as well as cytokines, oxygen, and nutrients can be transported, while waste products can be removed from the scaffold. Altogether, that kind of construction leads to excellent vascularization and osteogenesis within the scaffold [137].

Scaffolds with AVB were combined with rat ADSCs previously subjected to in vitro endothelial differentiation. Obtained ECs were incorporated into porous nano-hydroxyapatite-polyamide 66 (nHA-PA 66) scaffolds in vitro [111]. After that, AV bundle was inserted into ECs-based nHA-PA 66 in vivo. Also, AV bundle was inserted into nHA-PA 66 scaffolds seeded with non-induced ADSCs and into empty nHA-PA 66 scaffolds, while one experimental group had nHA-PA 66 scaffolds without inserted AV bundle and without cells. Two and four weeks after the implantation procedure, the implants were extracted from the animals and analyzed. Density of blood vessels was significantly higher, and the diameter of blood vessels was larger in ECs-based nHA-PA 66 scaffolds than that in all other groups of implants.

5.3.5 Gene therapy

Combining ex vivo gene therapy with cell transplantation techniques that include endothelial cell line is another approach for overcoming the problem of insufficient vascularization in bone. The benefit of this method was assessed by using 3D poly (lactide-co-glycolide) sintered microsphere scaffolds in a BTE approach [138]. ADSCs were isolated from human infrapatellar fat tissue, and the cells were transfected with adenovirus that encodes cDNA of VEGF and combined with endothelial ones. As a result, genetically modified ADSCs combined with ECs caused prominent growth within 3D poly (lactide-co-glycolide) scaffolds, which indicates the potential for ADSCs application in improving vascularization in BTE. Another study where gene therapy and ADSCs were combined was conducted by Peterson and associates [139]. First, ADSCs were transfected with the BMP-2 gene, then loaded on the collagen-ceramic carrier, and finally implanted in critical-sized femoral defect of nude mice. Eight weeks after implantations, histologic, radiographic, and biomechanical analyses showed that the collagen-ceramic carrier combined with ADSCs previously transfected with the BMP-2 gene caused bone formation within the defect.

Acknowledgements

The study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (project III41017).

Conflict of interest

The authors declare no conflict of interest.

Author details

Stevo Najman[1*], Jelena Najdanović[1] and Vladimir Cvetković[2]

1 Department of Biology and Human Genetics, Department for Cell and Tissue Engineering, Faculty of Medicine, University of Niš, Niš, Serbia

2 Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Niš, Serbia

*Address all correspondence to: stevo.najman@gmail.com; stevo.najman@medfak.ni.ac.rs

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