Biological characterization of mesenchymal stem cells (MSCs) isolated from ovine bone marrow and evaluation of the effectiveness of using MSC-enriched bioimplant in reconstruction of large bone defects in an experimental sheep model

The number of people suffering from bone injuries caused by traffic accidents, osteoporotic fractures, skeletal diseases, or tumor resections, is steadily increasing worldwide. Despite the fact that bone tissue has the ability to self-renew, about 10-20% of fractures heal abnormally or not at all. These abnormalities can lead to critical-sized bone defects, more than 1-2 cm long, covering more than half of the bone's diameter. At present, they remain the most difficult problem in orthopedic surgery, as well as a huge economic burden on the health care system, despite modern treatments with high technological standards.

Modern therapies for large bone defects are based on bone grafts, including autologous, allogenic and synthetic grafts. However, for critical-sized bone defects, autologous bone grafts are inapplicable due to limited bone supply, while readily available allografts can induce an immune response. Synthetic grafts, on the other hand, are freely available, but the process of osteointegration of the implant is slowed down, and the material itself lacks osteogenic properties. Consequently, new alternative treatments are being sought, among which a promising approach is the use of tissue engineering constructs, consisting of a bioimplant with osteogenic cells and biologically active agents.

The end of the last century brought a rapid development of research on the biology of mesenchymal stem cells (MSCs) and their potential application in regenerative medicine. MSCs have attracted an attention because of their ability to undergo multilineage differentiation, including osteogenic differentiation. However, regeneration of large bone defects using only cell therapies often fails because there is lack of matrix on which the cells can form 3D structures, similarly as in their natural tissue environment. This problem can be solved by creating a tissue engineering construct consisting of a biocompatible cell scaffold and MSCs with enhanced osteogenic potential.

The objectives of the doctoral dissertation were: 1) biological characterization of ovine bone marrow-derived mesenchymal stem cells (BM-MSCs); 2) evaluation of the effect of osteogenic stimulation of BM-MSCs with the cytokines FGF-2 and BMP-2; 3) characterization of the osteogenic properties of the scaffold-BM-MSCs construct stimulated with FGF-2 and BMP-2 *in vitro*; 4) evaluation of the biocompatibility and osteogenic potential of the scaffold-BM-MSCs construct after heterotopic implantation into tissues in the ovine mandibular region.

The dissertation is a series of thematically related works in the form of three scientific publications. The results of the research are presented in two original articles, while the review publication summarizes the current state of knowledge on the use of MSCs, scaffolds and biologically active factors in bone tissue regeneration.

The first publication (IJMS, 2020, 21(24):9726) includes studies of the biological properties of sheep BM-MSCs, which have been insufficiently characterized to date. In addition, the effect of FGF-2 and BMP-2 on the osteogenic potential of BM-MSCs *in vitro* was investigated. Baseline studies included evaluation of the phenotype, morphology, proliferation, ability to differentiate into bone, cartilage and adipose tissue cells, and secretory profile of ovine BM-MSCs under control culture conditions and after stimulation with FGF-2 or FGF-2 in combination with BMP-2. To investigate the effect of cytokines on the ability of BM-MSCs to differentiate into bone tissue cells, the presence of osteogenesis-related proteins collagen type I and osteocalcin was assessed by immunofluorescence staining, and the mRNA expression levels of genes encoding proteins of the early stages of osteogenic differentiation were analyzed: BMP-2, Runx2, osterix, type I collagen, and late markers of osteogenesis: osteocalcin and osteopontin. MSCs isolated from sheep bone marrow, preconditioned with FGF-2 and BMP-2, were shown to maintain their primary MSCs properties, as evidenced by the presence of surface markers CD73, CD90 and CD105 and the absence of expression of hematopoietic cell markers CD34, CD45 and major histocompatibility complex class II (DR) antigen. Stimulation with cytokines resulted in a more efficient proliferation rate, compared to untreated cells. Synergistic pro-osteogenic effects of FGF-2 and BMP-2 on BM-MSCs were observed, confirmed by alizarin red staining of calcium deposits and increased fluorescence intensity of osteogenic proteins: collagen I and osteocalcin. In addition, stimulation of BM-MSCs with cytokines FGF-2 and BMP-2 induced an increase in mRNA expression levels of genes of all analyzed osteogenic markers. It was also shown that sheep BM-MSCs produced biologically active factors, including those involved in osteogenesis (e.g., decorin), and stimulation of the cells with FGF-2 and BMP-2 modulated their secretory profile.

In the second publication (Cells, 2022, 11(21):3446), a tissue engineering construct consisting of a PCL/HAP/β-TCP scaffold coated with a n-HAP layer (fabricated at the Department of Materials Science and Engineering, Warsaw University of Technology) and sheep BM-MSCs treated with/or without FGF-2 and BMP-2 was developed. The effects of FGF-2 and BMP-2 on the osteogenic potential of cells cultured on the scaffold were studied *in vitro* and *in vivo* in an experimental sheep model. *In vitro* studies showed that preconditioning

the cells with FGF-2 and BMP-2 increased their ability to deposit and proliferate on the scaffold. Using alizarin red staining, assessment of ALP activity and expression levels of osteogenic markers, BM-MSCs cultured on the scaffold and treated with both cytokines were shown to have a significantly higher capacity to differentiate into bone tissue cells than in the absence of culture medium supplementation. The pilot *in vivo* studies (surgical procedures were performed at the Faculty of Veterinary Medicine, Wroclaw University of Life Sciences) have shown that the fabricated tissue engineering construct was biocompatible with sheep tissues and induced bone regeneration, as confirmed by the presence of the osteogenic proteins collagen type I and osteocalcin within the implanted bioimplant and the absence of elevated levels of pro-inflammatory cytokines in sheep serum after surgical operations.

The third publication (Cells 2021, 10(8):1925) describes the complex process of creating biologically active constructs for bone tissue engineering. The paper summarizes the latest developments in the use of MSCs, various types of scaffolds (ceramic, polymeric, or composite), and cytokines to promote osteogenesis in bone regeneration. In addition, examples of preclinical *in vivo* animal studies and clinical trials from the last decade are presented.

Studies conducted within this doctoral dissertation showed that stimulation of ovine BM-MSCs with FGF-2 and BMP-2 cytokines increased their osteogenic potential in 2D standard culture, as well as in 3D culture using a composite scaffold. The tested biomaterial promoted BM-MSCs adhesion and proliferation, and was biocompatible, as confirmed by *in vivo* studies. Pilot studies on implantation of the scaffold-BM-MSCs construct into sheep tissues indicated a beneficial effect of osteogenic induction in the area of the implanted bioimplant. In conclusion, the interdisciplinary research showed that the applied tissue engineering construct possesses pro-osteogenic properties and can bring the expected therapeutic effects in the repair of large bone defects.