Tapping the potential of bone regeneration by delivery of concentrated extracellular vesicles to the fracture site: A new horizon in bone regeneration therapy research?

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Abstract:

Background A small pilot study was conducted in order to investigate in situ regenerative potential of extracellular vesicles (EVs) in a rat subcutaneous implant model.
Material and methods An implant device was implanted in the axillary regions of four animals and consisted of a rat ABC enriched with an EV isolate obtained from human patients 7-21 days post bone fracture, or healthy individuals.
Results Upon histological examination of the implanted devices, there were clear evidence of early signs of immature cartilage and bone formation in the implants of fracture-EV treated animals, 21 days post implantation.
Conclusion These preliminary results suggest a potential for near-term innovation in the development of an affordable, non-invasive and completely autologous therapeutic device that can potentially enhance the existing regenerative capacity of skeletal tissues. EV can be used to concentrate the body's inherent regenerative potential and utilise it as a personalised self-healing therapy.

Keywords:

Extracellular vesicles, regeneration, subcutaneous assay, bone, fracture

Sažetak:

Istraživanje novih mogućnosti koštane regeneracije primjenom koncentrata izvanstaničnih vezikula na mjesto prijeloma: Novi pristupi u istraživanju terapije regeneracije kosti?
Uvod U navedenom istraživanju provedena je mala pilot studija kako bi se istražio in situ regenerativni potencijal izvanstaničnih vezikula (EV) u potkožnom štakorskom modelu.
Materijali i metode Nosač je implantiran u aksilarne regije životinja i sastojao se od autolognog krvnog ugruška (ABC) štakora obogaćenog izoliranim EV iz krvi pacijenata 7-21 dana nakon prijeloma kosti ili zdravih dobrovoljaca.

Rezultati Nakon histološkog pregleda implantata, potvrđeni su rani znakovi stvaranja nezrele hrskavice i koštanog tkiva u implantatima životinja koje su primile nosač i koncentrat EV nakon prijeloma, 21 dan nakon implantacije.
Zaključak Ovi preliminarni rezultati sugeriraju inovacijski potencijal u razvoju pristupačnog, neinvazivnog i potpuno autolognog nosača koji potencijalno može poboljšati postojeće terapije u regeneraciji kosti. EV nam omogućavaju da koncentriramo endogene molekule s regenerativnim potencijalom koje se mogu koristiti u personaliziranim terapijskim pristupima.

Ključne riječi: Izvanstanične vezikule, regeneracija, potkožni test, kost, prijelom

Introduction

Bone fractures often lead to loss of quality of life, long-term disability and increased mortality (1–3). They are a major economic burden on national healthcare systems, costing the European Union over €56 billion annually (4). Due to a globally ageing population with an increased incidence of osteoporosis, fracture-related burden is expected to increase significantly over the coming decades. Bone is an active organ that undergoes continuous lifelong remodelling and has a significant regenerative potential. Formation of new skeletal tissue involves three overlapping phases: inflammation, renewal and remodelling (5). Upon trauma, bone fragments tear the neighbouring blood vessels, triggering blood clotting, hematoma formation and inflammation. Within days, the fracture site progresses to a temporary fibrocartilaginous callus that is replaced over several weeks by a bony callus, that joins the broken ends. Finally, the newly formed bone is remodelled over a longer period (months to years) effectively erasing the breakage site (6,7). Standard therapy assisting fracture repair relies on mechanical support by plaster and/or mechanical devices (e.g. nails, plates and screws). Although effective, this passive immobilisation approach dates back to the beginnings of recorded human history (8,9) and in large bone defects and some pathological fractures, the underlying physiological bone repair is insufficient. Therefore there is a pressing medical need for new personalised therapies that enhance bone regeneration, shorten healing times and prevent bone non-unions (10). A common therapy in such cases is bone grafting, a procedure that includes highly invasive autologous bone transplantation and has a limited success rate (7). A more sophisticated approach encourages skeletal regeneration by deploying recombinant human bone morphogenetic proteins (BMP) 2 or 7 to the fracture site via a bovine collagen carrier. However, this method is plagued by low efficiency and severe side effects due to the immunogenic nature of bovine carriers (7). A second generation of similar devices utilises BMP6, a superior bone formation inducer, that is deployed using a non-immunogenic autologous blood coagulum (ABC). Since fracture repair starts with a blood clot, the ABC is an ideal vector, however, the production of clinical grade rhBMP6 is expensive, time consuming and complex (7,11). We propose a third generation of completely autologous, highly inductive endogenous materials that enhance the body's natural capacity for bone regeneration.

Extracellular vesicles (EVs) are lipid bilayer-delimited particles released by most cell types. They provide a readily available sample of the inner workings of the cell that are dependent on current (pato)physiological events or trauma. These vesicles shuttle a variety of proteins, lipids, nucleic acids (including mRNA and microRNA), and are an under-recognized form of intercellular communication (12,13). EVs are commonly divided by size into microvesicles (MVs) (200 nm-1 μm) and exosomes (50-200 nm), but a functionally more relevant classification is their location of origin (12,13). MVs are produced by direct outward budding of the plasma membrane, so their cargo is a fraction of the "mother" cells' cytoplasmic content (14). Conversely, exosomes are generated by the endosomal compartment in multivesicular bodies, and they therefore express typical endosomal markers and surface molecules that can target them to recipient cells (15,16). The fact that EVs can traffic a mixture of functional bioactive molecules and remotely influence recipient cells at the post-transcriptional level, has profoundly changed our understanding of gene regulation (14). Due to specific membrane markers, the vesicles are recognized and internalised by neighbouring or remote cells through receptor-ligand interactions, endocytosis and/or phagocytosis, or they can fuse with the target cell membrane and deliver their contents into the cytosol (17–19). Crucially, EVs isolated from different sources have different contents reflecting their current environmental conditions. Being derived from cells, these vesicles are biocompatible, stable and non-immunogenic and they present an ideal tool for different therapeutic strategies.

Participants and methods

Study outline

This prospective observational study was approved by the Ethics Committee of the Hospital Center "Sisters of Charity", University Hospital Center (SCUHC) Zagreb (EP-003-06/20-03/023) and the Ethics Committee of the School of Medicine, University of Zagreb (EP 331/2021) with informed consent obtained from the patients to use the samples for research purposes. Study outline is presented in Figure 1.

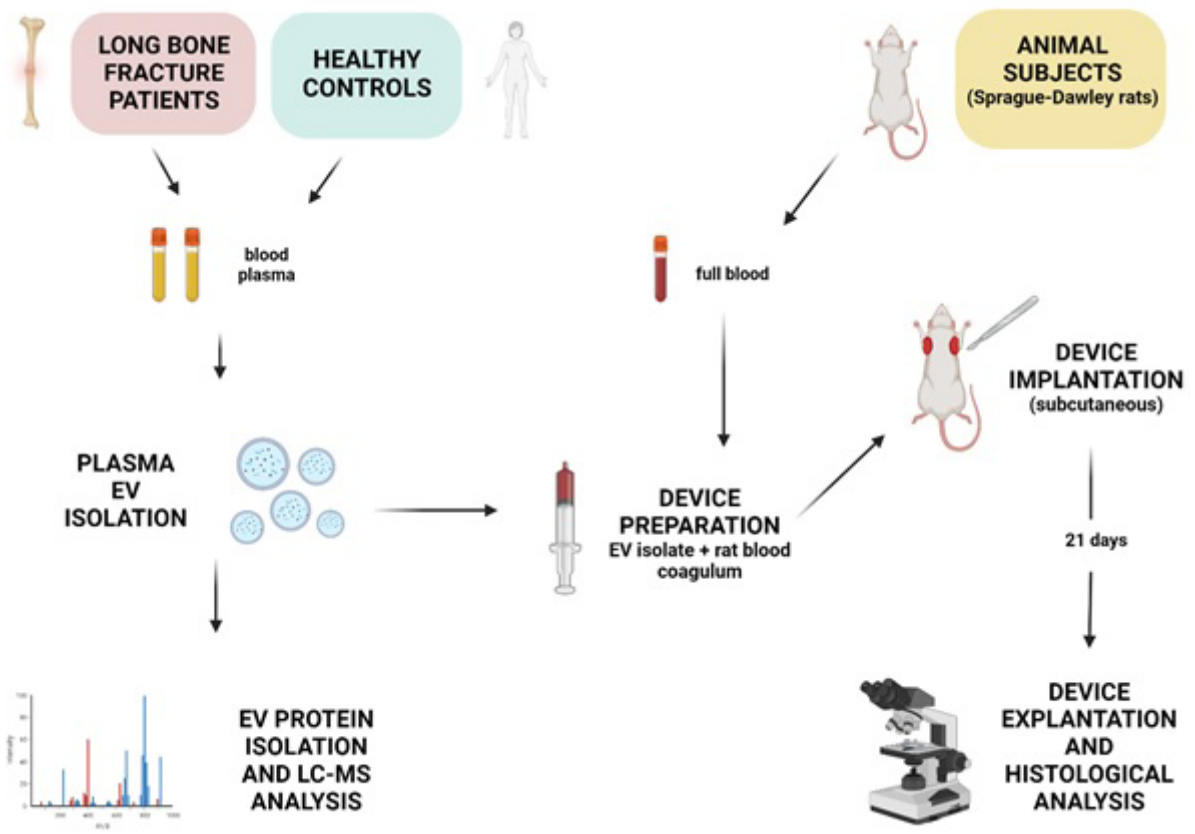

Figure 1. Study outline which consists of human plasma derived EV isolation, subcutaneous animal assay preparation and LC-MS analysis. Created with Biorender.com.

Human subjects

The study included two subject groups: 1) patients with a metaphyseal long bone fracture (N=15); and 2) healthy control group (volunteers; N=15) (Figure 1). Each participant in the study met the inclusion criteria, which included being within the age range from 18 to 70 years, possessing clinical and radiological confirmation of a fracture (for individuals in the fracture group). Exclusion criteria were malignancy, active infection, osteoporosis, or immunocompromised conditions. Blood samples were collected in the first group within the 7 to 21-day window following the occurrence of the fracture. This specific timeframe was chosen to capture the transition phase from inflammation to fibrocartilaginous healing, with the expectation that the patients would exhibit peak blood expression levels of growth and signalling factors during this period

Blood withdrawal

Withdrawal of 10 mL of venous blood from the patient of concern was obtained into the provided plain vacutainer which contains citrate as anticoagulant substance. Blood was then centrifuged at 1500 x g for 15 minutes in order to separate platelet-free plasma. The obtained plasma was separated and stored at -80°C until further analysis. Plasma samples were pooled into two above-mentioned groups; from which EVs were isolated by differential centrifugation (20). These pools were used for implant preparation and for proteomic analysis.

Animal subjects

This study included N=4, nine-month-old male Sprague-Dawley rats body weights between 350 to 400 grams from registered breeding facility School of Medicine, University of Zagreb, Department of Pharmacology, No: HR-POK-007 . As the main carrier, autologous blood (ABC) from each laboratory animal was used. Animals were divided into four groups: 1) positive control (BMP6 in ABC), 2) negative control (ABC only); 3) EV isolate from healthy individuals in ABC; 4) EV isolate from patients with long bone fractures in ABC, Table 2. Recombinant BMP6 (rhBMP6) obtained from R&D Systems was used for preparation of positive control devices.

Implant device preparation

Implant device (ABC) was prepared from 0.5 mL of full blood obtained from rat tail vein, which was mixed with an appropriate amount of EVs (isolated from 10 and 15 ml of human plasma) and left for 60 minutes to coagulate in a 1 mL syringe. After removing the serum, the coagulum (ABC) was ready for implantation.

Rat subcutaneous implant assay

EVs osteogenic activity in ABC was tested at different doses in the rat subcutaneous assay (Table 2). Animals were anaesthetised using injections of ketamine (60 mg/kg i.p.) and xylapan (20mg/kg i.p.). Anaesthesia was maintained with 2-3% of isoflurane. Animals received analgesic (carprofen 5-15 mg/kg s.c.) per day. A vertical incision (1 cm) was made under sterile conditions in the skin over the thoracic region, and the pockets were prepared by blunt dissection on both sides of the incision. A small pocket was created under the skin in the axial regions to implant the prepared device. After implantation the site was sealed with a single suture to the fascia and three sutures for the skin. The implant response of EVs was tested by histology analyses. The day of implantation was designated as day 0 of the experiment. Implants were removed on day 21 for analysis after the euthanasia procedure using 100 mg/kg of ketamine and 60 mg/kg of xylapan i.p. Laboratory animals were cervically dislocated to confirm the success of euthanasia method.

Histology

Histological examination was used to assess the extent of biological/regenerative activity in the implant. Tissue samples were collected and fixed in 10% formalin solution. Samples were cut at 5 µm slices and stained with hematoxylin-eosin and safranin staining methods. Stained slides were analysed, using an optical microscope (Zeiss Axiostar plus, Artisan technology group. magnification range 20x and 40x) by three independent researchers. Among them, one was an expert in animal histopathology.

Table 2. Animal subjects and implanted devices

Animal designation	Device components	Right axillary region	Left axillary region
1 - Positive control	Inductor	1 µg rhBMP6	2µg rhBMP6
	Carrier	0,5 mL of rat-1 blood	0,5 mL of rat-1 blood
2 – Negative control	Inductor	100 µl saline	150 µl saline
	Carrier	0,5 mL of rat-2 blood	0,5 mL of rat-2 blood
3 – EV isolate from healthy individuals	Inductor	100 µl of EV isolate derived from 10 mL of healthy individuals' plasma	150 µl of EV isolate derived from 15 mL of healthy individuals' plasma
	Carrier	0,5 mL of rat-3 blood	0,5 mL of rat-3 blood
4 – EV isolate from post-fracture patients	Inductor	100 µl of EV isolate derived from 10 mL of patients' plasma	150 µl of EV isolate derived from 15 mL of patients' plasma
	Carrier	0,5 mL of rat-4 blood	0,5 mL of rat-4 blood

Characterization of EV proteins

After EV isolation (20), vesicle membranes were lysed by sonication (5 min / 75% amplitude). The released proteins were precipitated by acetone (1 hour / -80°C), centrifuged (10 min / 16000 g) and resuspended in 8 M urea. Protein concentrations of the were determined by Lowry assay (BioRad RC DC Proteins Assay) and a plate reader (*SpectraMax i3x - Molecular Devices LLC.*).

Protein pools (40 µg) were further processed 10-kDa centrifugal filter units. After alkylation with 55 mM iodoacetamide in 8 M urea (20 min / RT / dark) and digestion by 0.8 µg of trypsine (ON / 37 °C; Worthington, TPCK treated), the obtained peptides were purified using stage tips (21). Peptides were then separated on a 15 cm C18 nano-column by HPLC (Ultimate 3000, Thermo Fischer Scientific) and injected to an LTQ Orbitrap Discovery (Thermo Fischer Scientific) mass spectrometer. Raw data was processed using MaxQuant software version 1.5.1.2. (Max Planck Institute of Biochemistry) using the default settings. Functional enrichment of the identified proteins across sample pools was performed by FunRich software 3.1.3 by using quantitative analysis and by using the FunRich protein database.

Enrichment of biological pathways was analysed by comparing the dataset of identified proteins in the bone fracture group against the healthy control group. Biological pathways relevant for bone metabolism were selected by manual curation guided by extensive literature search. Samples were analysed in technical triplicates.

Results

EV protein cargo analysis

Analysis of the protein cargo of isolated EVs yielded 28 identified proteins in the bone fracture group and 30 proteins from the healthy control group. The datasets of identified proteins were analysed for common proteins and outliers (Figure 2). We found that there were 17 commonly identified proteins in both groups, and a substantial number of proteins expressed only in the bone fracture and control groups (outliers), 11 and 13, respectively. The dataset of identified proteins was also analysed by comparing the bone fracture group against the control group for enrichment of relevant biological pathways (Figure 3). We found that the proteins identified in the fracture group were enriched in several pathways such as cell-cell communication, cell-ECM communication, Wingless-related integration site (Wnt) signalling network, Platelet-derived growth factor (PDGF) receptor, Vascular Endothelial Growth Factor (VEGF) and Granulocyte macrophage colony-stimulating factor (GMCSF) signalling network and Insulin growth factor 1 (IGF1) pathway.

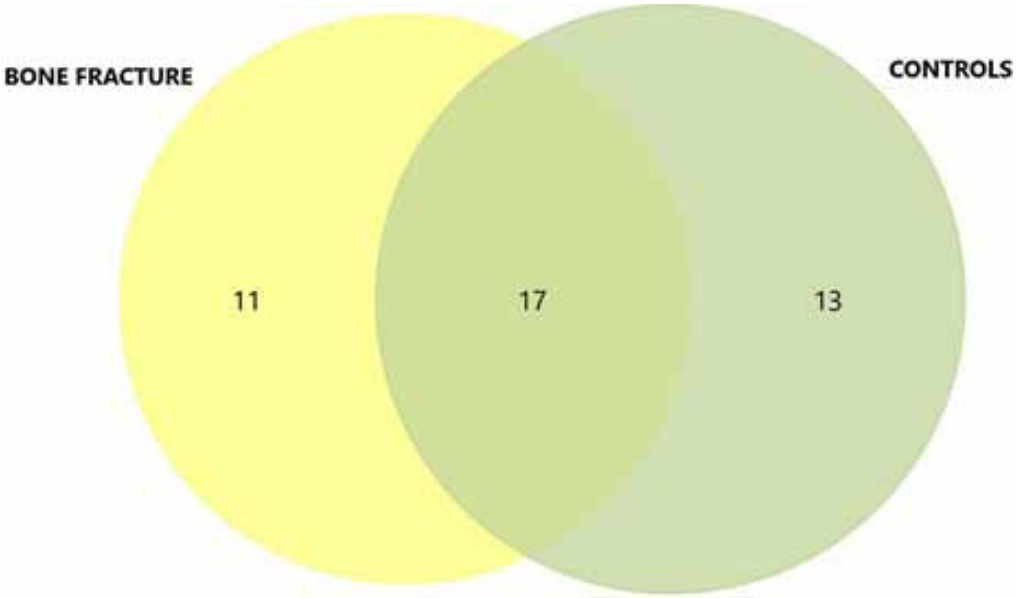

Figure 2. Venn diagram showing identified overlap of identified EV proteins and proteins identified only in one group.

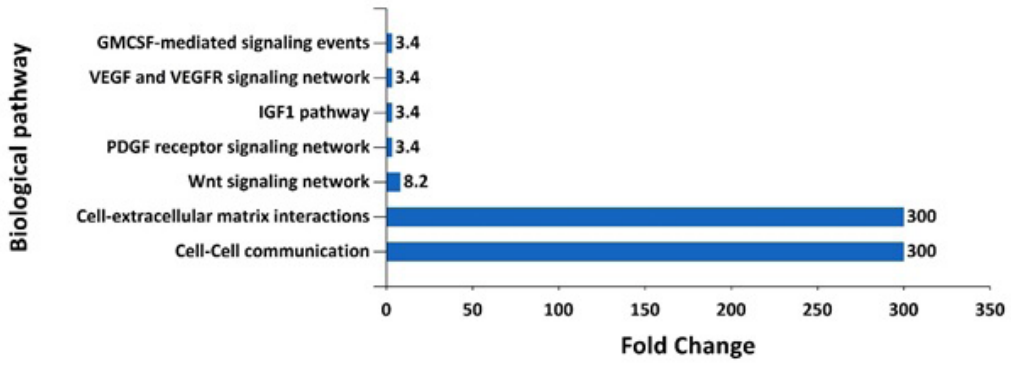

Figure 3. Bar chart showing fold enrichment in a selection of relevant biological pathways in the bone fracture group compared to the healthy control group.

Histological evaluation of autologous carrier/EV implants

Histological analyses of implants which contained EV isolate from long bone fracture patients, explanted from rats after 21 days and stained by HE revealed that a certain level of cell grouping was observed. While normal structure of surrounding connective tissue was not disturbed, regions of implantation were marked by presence of cells which formed rather distinguishable islets (Figure 4). Interestingly, in the case of larger islets, a gradient from outside towards the inner region of the islets in some morphological parameters was visible: cells in the outer zone of the islets were marked by a smaller amount of extracellular matrix (ECM). Cells in the middle of larger islets are surrounded by a large amount of ECM and some of them are clearly characterised by smaller nuclei (see Figure 4; A,B asterisk). Notably, these results were not observed in implants containing EV isolate from healthy volunteers, wherein remnants of the coagulum carrier were still evident (see Figure 4C, black arrows). Additional staining with safranin, which is selective for cartilage-typical proteoglycans, revealed that islets do contain molecules which safranin recognizes (Figure 4, D red colour). Implants with BMP6 as positive control demonstrate mineralized bone with a bone marrow and absence of inflammation and fibrosis at the external surface of the implant (data not shown).

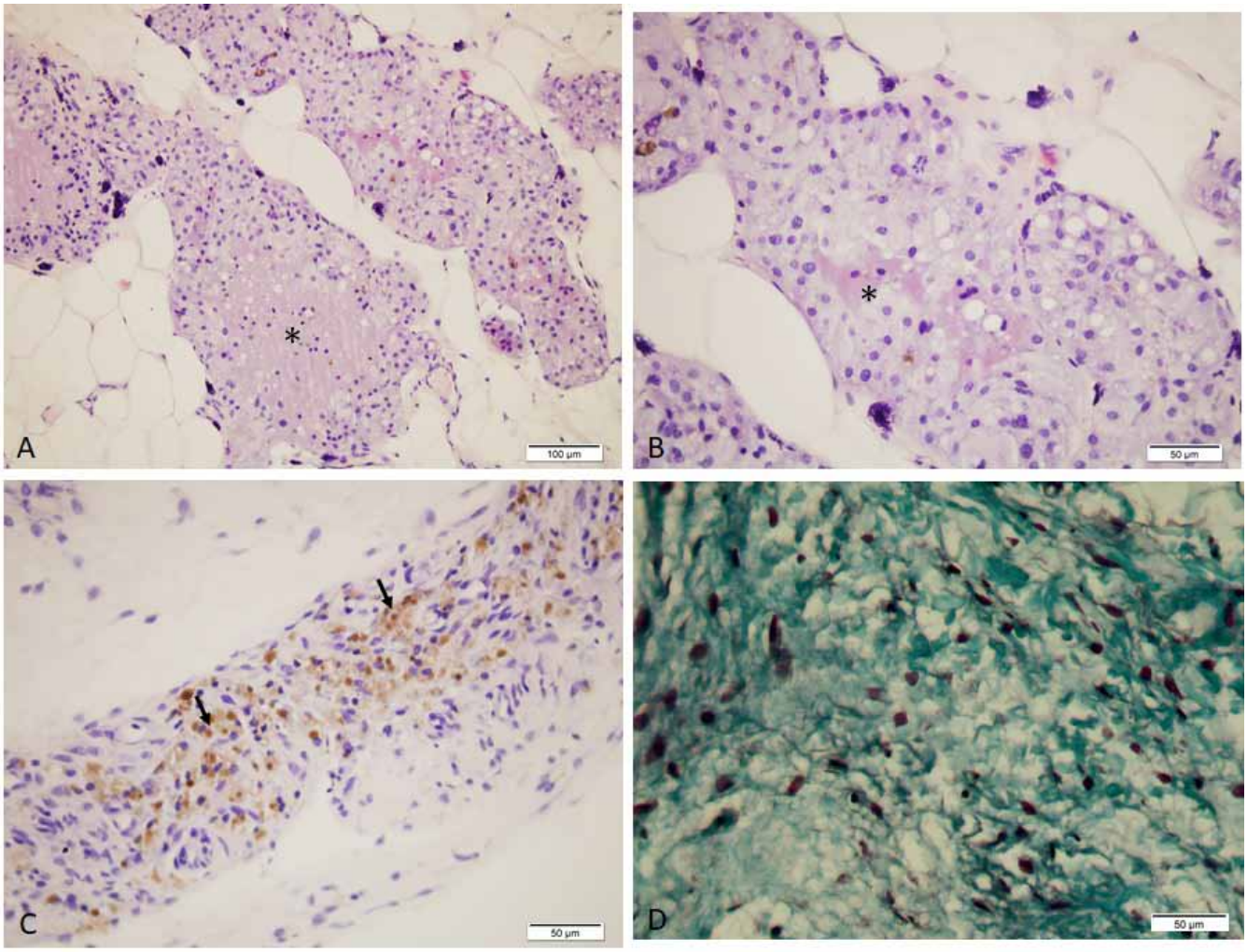

Figure 4. Histological analysis of implant after 21 days following implantation. A) H&E staining of implant enriched with ECVs of patients with fracture (magnification 20x); B) H&E staining of implant enriched with ECVs of patients with fracture (magnification 40x). Cells in the middle of larger islets are surrounded by large amount of ECM and some of them are clearly characterised by smaller nuclei (asterisk); C) H&E staining of implant enriched with ECVs of healthy volunteers (magnification 40x) where remnants of autologous blood coagulum are visible (black arrows); D) safranin red staining of implant enriched with ECVs of patients with fracture (magnification 40x)

Discussion

The need for personalised therapeutic regenerative approaches is significant. In this small pilot study, we aimed to demonstrate the endogenous regenerative potential of EVs for the purpose of improving and developing new therapeutic solutions, particularly in the field of bone and cartilage regeneration. Our goal was also to show a significant shift in the different molecular profiles between healthy individuals and individuals with bone fractures by analysing the proteomic profile of vesicles and their biological impact.

Our histology analysis results indicate the regenerative potential of EVs in the direction of tissue regeneration. To some extent this resembles a normal differentiation pattern of bone formation, especially during intramembranous ossification (forming of flat bones): while cells at the edge of the islet are metabolically very active (osteoblasts), cells which remain trapped inside the islet are surrounded by larger amount of ECM, alongside metabolic downregulation which is as well characterised by smaller diameter of nuclei (osteocytes). Another process of differentiation in the connective tissue which might be, in some elements present here is differentiation of hyaline cartilage, which is also marked by more dense packed cells at the edge (chondroblasts) and cells trapped within a huge ECM forming territory (chondrocytes). Since surrounding tissue stained by safranin did not exhibit any significant positivity, this is an additional proof that the observed islets are composed of cells which undergo a certain level of differentiation and secretion of ECM highly resembling the one normally found in either bone or cartilage. It is necessary to increase the number of samples and include additional histological staining methods to fully ascertain the direction and dosage critical for initiating differentiation. A dose-dependent effect of ECV was not observed, therefore, the range of implanted ECV dose should be expanded.

Our proteomic analysis of EV cargo further strengthens our histology findings. Namely, our gene enrichment analysis revealed a clear distinction in proteomic profiles between EV isolates of long bone fracture group when compared to healthy volunteers. We found several pathways which are known to play vital roles in bone formation and remodelling, including cell-cell and cell-ECM communication, Wnt signalling network, PDGF receptor, VEGF and GM-CSF signalling networks, as well as activity in the IGF1 pathway (22–28). Taking both the histological and proteomic disparities between the two groups into account, it is likely that specific EV-derived molecular factors associated with fracture repair play a pivotal role in triggering bone regenerative potential. These findings further highlight the potential EV-based therapies might play in the field of bone regeneration therapies.

There are three major limitations of the present study. The used Sprague-Dawley rats were adult animals (9 months old) with a decreased regenerative capacity in comparison to the commonly used two month-old animals that are at peak age for bone regeneration (29). This was a deliberate choice, i.e. a built in fail-safe in order to more strictly test the bone-induction potency of EVs. Secondly, the present study describes the regenerative potential of human EVs delivered in a rat ABC. Although the mechanism of bone regeneration was not scrutinised, plausibly, it is mediated through regulatory microRNA and signalling pathways included in bone metabolism. Although microRNA, as well as some of the bone morphogens (eg. BMP family) are evolutionarily conserved, it is expected that human bone tissue would respond better to stimulation by human EVs (30,31). However, due to their heterogeneity and small size, the isolation and analysis of EVs is not standardised and a variety of isolation and detection methods are available (19). The role of EVs in health and disease is emerging, and their biomarker and therapeutic potential appear promising across almost all biomedical fields (32–34).

Conclusions

The hypothesis derived from this preliminary pilot study provides novel insights, indicating that EVs may potentially exhibit regenerative efficacy, particularly through refinement of dosage titration. Consequently, our forthcoming actions will focus on augmenting the quantity of EVs and investigating a diverse array of biological combinations. These initiatives form an integral part of our ongoing commitment to advancing the development of personalised self-healing therapy.

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Conflicts of interest

The authors declare no conflicts of interest

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee of the Hospital Center "Sisters of Charity", University Hospital Center (SCUHC) Zagreb (EP-003-06/20-03/023) and the Ethics Committee of the School of Medicine, University of Zagreb (EP 331/2021).

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