Paracrine Effect of Mesenchymal Stem Cells derived from Human Adipose tissue in Bone Regeneration.

By Linero I., Chaparro O., 2014

Background

Background

- Stem Cells:
  - undifferentiated cells that are able to differentiate into specialized cells
  - Classical abilities: self-renewal and potency
- Two types of stem cells:
  - embryonic stem cells →isolated from blastocysts (4-5 days post fertilization
  - adult stem cells → in various tissues →"repair system of the body"
    - Sources of autologous adult stem cells: bone marrow, adipose tissue, periosteum, blood, umbilical cord
- Application:
  - Bone marrow transplant → for patients with cancers of blood or bone marrow (multiple myeloma or leukaemia)
    - Problems:immunosuppression, no specific cell type →pluripotency, some stem cells form tumors

Background

- Mesenchymal Stem Cells (MSC) in Research and clinical practice:
  - initially isolated from bone marrow → adipose tissue is now the best option for clinical applications → only minimal invasive methods are needed for it
  - MSC application of musculoskeletal injuries
    - previous thought: MSC would differentiate into the needed cell type after implanting them
    - now: the paracrine effect of the MSC is the primary mechanism of their therapeutic effect→ secrete biologically active molecules that brings beneficial effects on injured tissues by stimulating angiogenesis and tissue regeneration and inhibits fibrosis, apoptosis and inflammation

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Background:

- Mesenchymal Stem Cells (MSC) in Research and clinical practice:

  - supported in vitro and in vivo studies which showed cells responding to paracrine signaling from MSC → survival, proliferation, migration and gene expression

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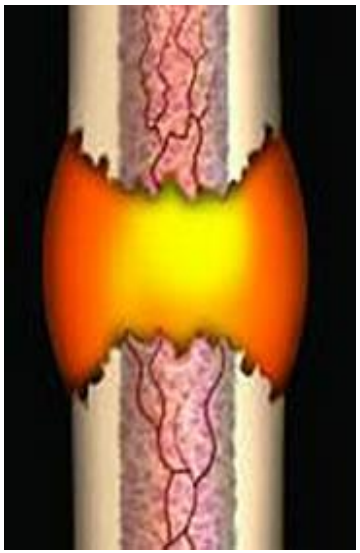
Background

- **Bone healing:**
  - proliferative physiological process
  - factors, that affects the healing process:
    - Age, Bone type, drug therapy and pre existing bone pathology, nutrient intake
  - Treatment of bone fractures:
    - relocation of fractures, immobilization and surgery
  - mainly influenced by the periosteum (the connective tissue membrane covering the bone) → source of precursor cells (chondroblasts and osteoblasts)

Background

- **Bone healing:**
  - bone marrow (if present), endosteum, small blood vessels, and fibroblasts provides other sources of precursor cells
  - has 3 overlapping stages:
    - **inflammatory phase:**
      - Starts after the bone fracture and lasts for 5 -7 days
      - because of the good blood supply of bones the fracture bleeds
      - the extravascular blood cells form a blood clot → haematoma → template for the callus
      - Blood cells and macrophages release TNFα, IL-1, IL-6, IL-11, IL-18
      - TNFα mediates through Tumor necrosis factor receptor 1 (TNFR1) and Tumor necrosis factor receptor 2 the differentiation of MSC into osteoblasts and chondrocytes

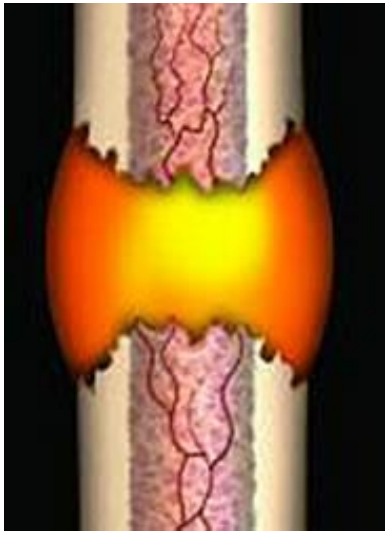


www.physioroom.com/injuries/bone_fracture/3_inflammation.php

Wikipedia
https://www.foothealthfacts.org/conditions/bone-healing

Background

- Bone healing:
  - **inflammatory phase:**
    - MSC are recruted by Stromal cell-derived factor 1 SDF-1) and CXCR4 (**chemokine receptor type 4)**
    - IL-1 helps to form the callus and the formation of blood vessels
    - IL-6 promotes the differentiation of osteoblasts and osteoclasts
    - cells die within the blood clot and fibroblasts replicate
    - form a granulation tissue
    - osteoclasts remove the dead cells



www.physioroom.com/injuries/bone_fracture/3_inflammation.php

Wikipedia
www.foothealthfacts.org/conditions/bone-healing

Background

- Bone healing:
  - **<u>Bone production:</u>**
    - After 7 – 9 days the periosteum provides periosteal cells
    - Periosteal cells proximal to the fracture →chondroblasts →form hyaline cartilage
    - Periosteal cells distal to the fracture →osteoblasts → form woven bone → through bone resorption of calcified cartilage and recruitment of bone cells and osteoclasts
    - finally this two tissues unite with each other → form the fracture callus → gap is bridged

www.physioroom.com/injuries/bone_fracture/4_soft_callus_formation.php

Wikipedia
www.foothealthfacts.org/conditions/bone-healing

Background

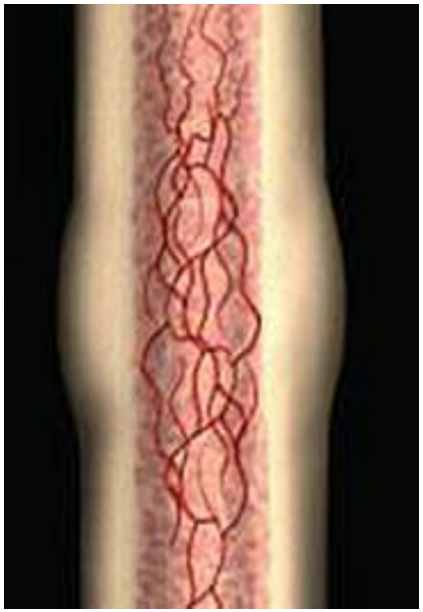
- Bone healing:
  - **<u>Bone production:</u>**
    - endochondral ossification → replacement of the hyaline cartilage and woven bone with lamellar bone
    - induced by IL-1 and TNFα
    - The collagen matrix becomes mineralized →is penetrated by microvessel and osteoblasts
    - osteoblasts form new lamellar bone upon the surface of the mineralized matrix
    - So the the woven bone and cartilage is replaced by trabecular bone

www.physioroom.com/injuries/bone_fracture/4_soft_callus_formation.php

Wikipedia
www.foothealthfacts.org/conditions/bone-healing

Background

- Bone healing:
  - **<u>Bone remodeling:</u>**
    - starts 3 – 4 weeks after fracture and can last 3 – 5 years to be completed
    - osteoclasts resorb trabecular bone and then osteoblasts replace it with compact bone
    - Bone remodelled into the old shape as before the fracture



www.physioroom.com/injuries/bone_fracture/4_soft_callus_formation.php

Wikipedia
www.foothealthfacts.org/conditions/bone-healing

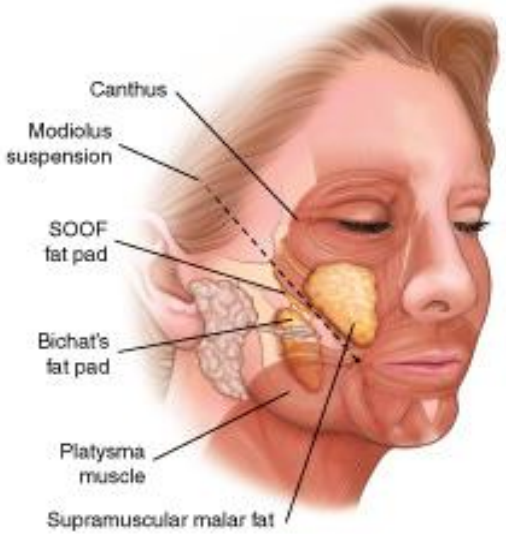
Background

- Bone healing:
  - **<u>Complications:</u>**
    - Infection (post-traumatic wound infection, chronic osteomyelitis)
    - Non-union (no healing within 6 months, caused by infection or lack of blood supply)
    - Mal-union (deformation, most common in long bones such as the femur)
    - Delayed-union (slower healing, persistence of the fracture line and a scarcity or absence of callus formation on X-ray

Methods

Methods

- <u>Isolation of Mesenchymal Stem Cells (MSC):</u>
  - MSC from adipose tissue samples
  - obtained from a biopsy of the Bichat`s fat pad of a 23 year old female person
  - Bichat`s fat pad → one of the encapsulated fat masses in the cheek



www.pinterest.de/pin/80431543322483745/

Methods

- Culture od MSC:
  - Tissue explants of the adipose tissue → planted in 6-well culture plates with :
    - 2 ml of Dulbecco's Modified Eagle's low glucose medium,
    - 10% fetal bovine serum (FBS, Invitrogen)
    - penicillin 100 U/ml and streptomycin 100 mg/m
    - Incubated in 37˚C in a humid atmosphere with 5% CO2
    - Twice a week half of the medium was replaced with a fresh one until the cell population grew 70 – 80%

Methods

- Preparation of the conditioned Medium:
  - After letting MSC grow to 70 – 80 % they were washed twice with 1X Phosphate Buffered Saline (PBS) and incubated in serum-free medium (OPTIMEM) unter hypoxic condition (2% O2) for 24 h.
  - Then the medium was cleared by a centrifugation
  - the protein concentration adjusted with serum-free medium (OPTIMEM) to 100 (CM-1) and 200 mg/ml (CM-2)
  - Sterilized by a syringe filter

Methods

- Characterization of Condition Medium (CM):
  - It was evaluted to detect 43 human proteins (cytokines, growth factors, proteases and soluble receptors) with Human Angiogenesis Antibody Array C1000
  - 1 ml of CM was incubated with arrayed antibody membranes for 2 h
  - The membranes were washed and then icubated for 1 h with the mixture of biotin-conjugated antibodies
  - HRP-conjugated streptavidin was added for 1 h after the washing
  - For the signaling dection buffer was added and the mebranes were exposed to autoradiographic films.

Methods

- Preparation of Human Blood Plasma Hydrogels (HBPH):
  - Plasma samples were obtained by the same female individual
  - After mixing human blood plasma1X PBS, Tranexamic Acid, Calcium Chloride 1% (CaCl2) and Dulbecco's Modified Eagle's medium low glucose , they gelated it.
  - because of being transparent and having homogeneous composition, without sediments, it is possible to microscopic visualize the MSCs during proliferation and differentiation.

Methods

- Animal model:
  - 19 male New Zealand white rabbits
  - in the mandibular angles bilateral and bicortical surgical defects of 10 mm diameter were created
  - Divided in 3 groups:
    - 1.group: 12 rabbits → on one side HBPHs with Ad-MSC and on the other side HBPHs without cells
    - 2.group: 4 rabbits → on both sides with HBPHs with Ad-MSC and sacrificed at 3, 6, 9 and 12 days after the implantation
    - 3.group: 3 rabbits → hydrogels containing CM-1 on one side and CM-2 on the other side

    (protein concentration adjusted with serum-free medium (OPTIMEM) to 100 (CM-1) and 200 mg/ml (CM-2) after centrifugation)

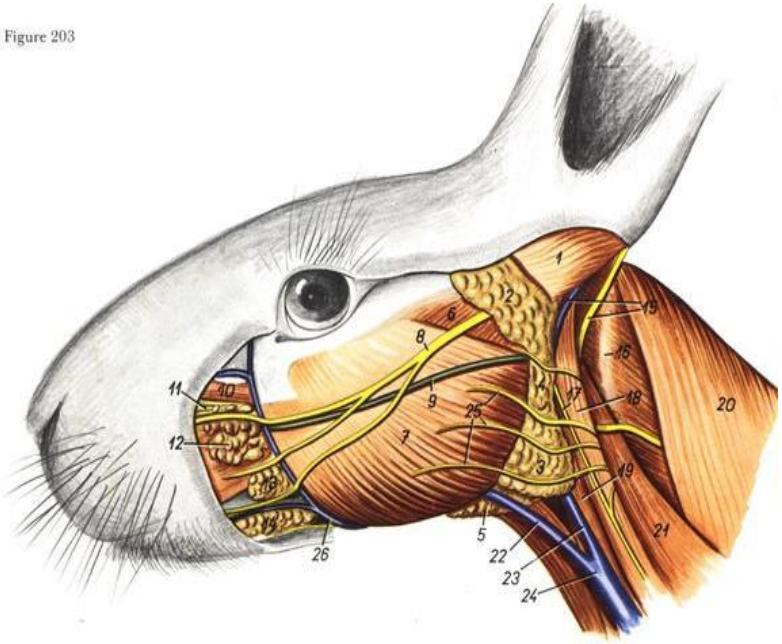
Methods

- Surgical Procedure:
  - Anesthesia: intramuscular injection of xylazine (3 mg/kg) and ketamine (20 mg/kg)
  - at the site of incision local anesthesia of a 2% Lidocaine with 1:80 000 adrenaline was given regularly.
  - 1 h prior to surgery an intramuscular injection of benzathine penicillin was given.
  - After preparing the surgical area by shaving the skin and disinfecting it, the mandibular angle was exposed by detaching the masseter on the buccal and lingual side.

Methods

- in the anterior region to the mandibular angles slowly rotating trephine bur (SALVIN) was used to do circular defects 10 mm in diameter
- The critical size defect → which prevents spontaneous healing during the animal lifetime → 5mm in this animal model!
- Hydrogels were implanted based on the treatment!
- Soft tissues where repositioned and sewed.

Methods



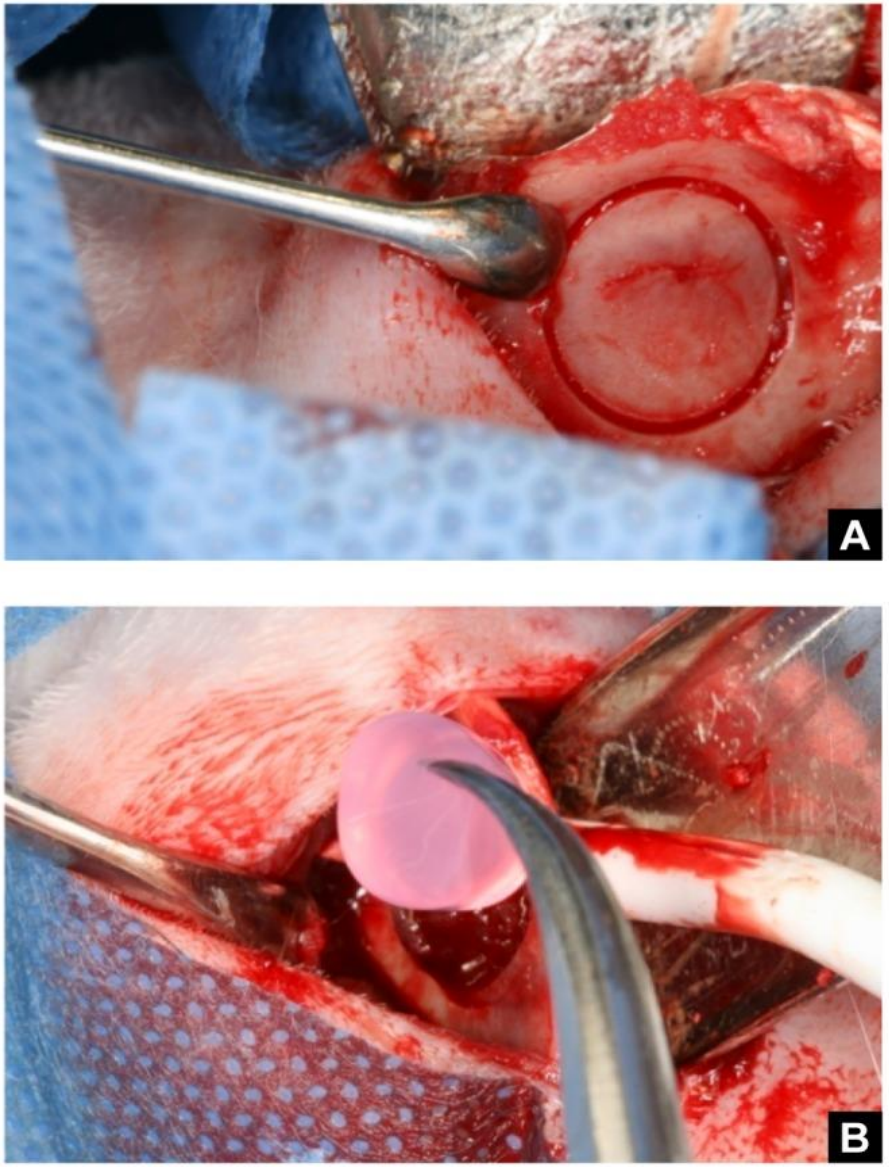


Figure 1. Surgical procedure. A. Circular demarcation of bone defect. B. Implantation of HBPH.

https://hiveminer.com/Tags/anatomy%2Crabbit/Recent
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Methods

- Radiographic Analysis:
  - postoperative skull radiographs
  - The inital defect was compared to the newly formed bone tissue
- Morphometric Analysis:
  - Surgical specimen was obtained postmortem
  - photographs were taken and the percentage of newly formed bone tissue estimated over the time using the Image J 1.410 program

Methods

- Histological Analysis:
  - Surgical specimen were:
    - fixed by immersion in 10% formaldehyde solution for 72 h
    - decalcified in Shandon TBD-1 Rapid Decalcifer for 24 h
    - dehydrated in ascending series of ethanol
    - embedded in paraffin
    - were stained with hematoxylin-eosin and blue toluidine to locate condroitin sulfate in the cartilage matrix and and intramembranous bone
    - They used Massontrichome to identify collagen fibers and calcification process

Methods

- To detect Ad-MSC:
  - Specimen of the mandibular were fixed in 10% formaldehyde for 72 h
  - Decalcified in EDTA for 2 months
  - Dehydrated in ethanol
  - Put into a xylene-solution of 100% for 30 min.
  - Embedded in paraffin
  - Cut into 4 µm section for immunhistochemical detection of human beta-2-microglobulin positive cells

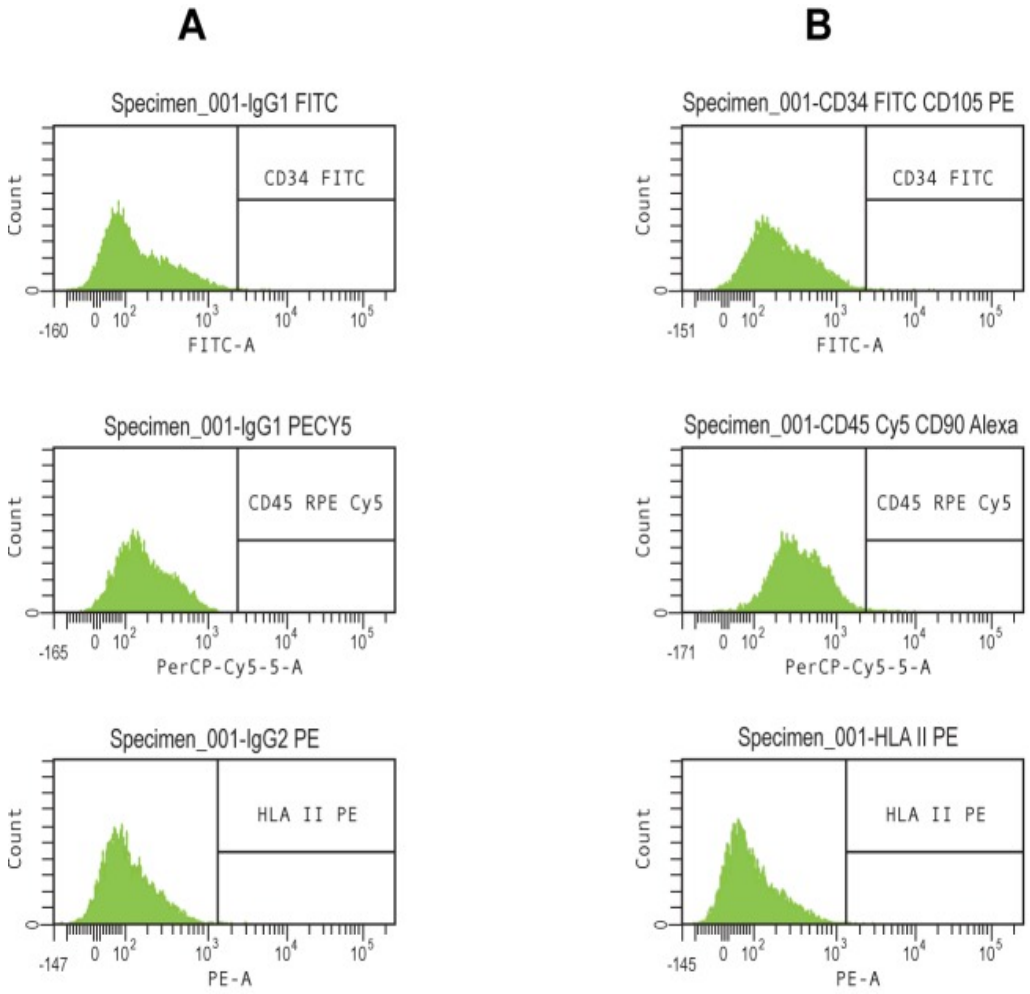
Methods

- Immunhistochemistry:
- To track Ad-MSC:
  - Sections were deparaffinized
  - Incubated with horse serum
  - Treated with anti-human beta-2 microglobulin-HRP primary antibody for 1 h
  - Incubated with biotinylated secondary antibody for 30 min.
  - Revealed with diaminobenzidine.

Results

Results

Figure 2: Ad-MSC characterization: Flow Cytometry



Flow cytometry determined the phenotype of Ad-MSC

A: Isotype controls for each marker

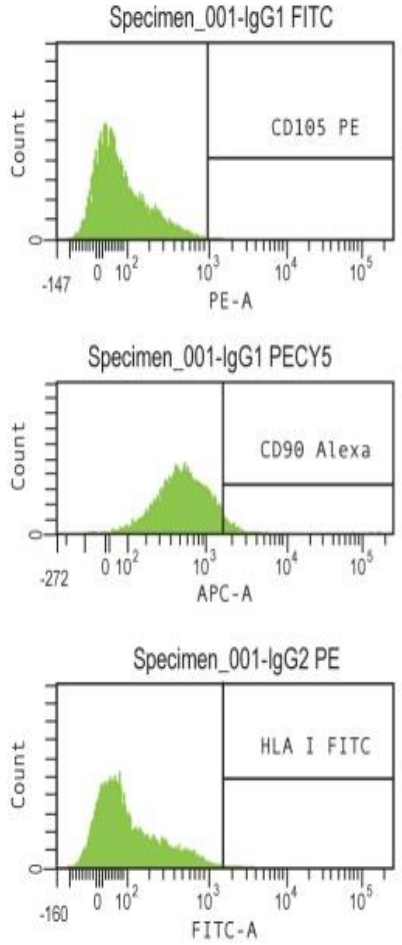
B: Ad-MSC labeled with CD34-FITC, CD45-RPECy5, HLA II-RPE, CD105-PE, CD90- Alexa, HLA I-FITC.

Ad-MSC:
→negative for CD45, CD34, HLA II

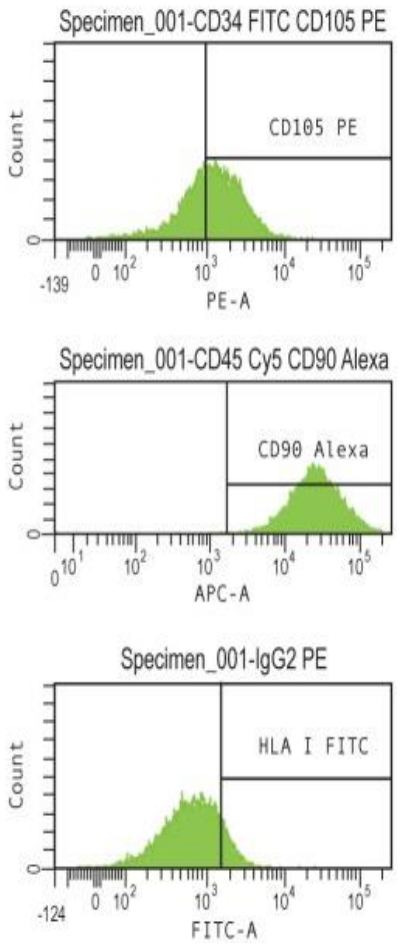
Results

Figure 2: Ad-MSC characterization: Flow Cytometry

A



B



Ad-MSC:

→ positive for CD90, CD105, HLA I

A

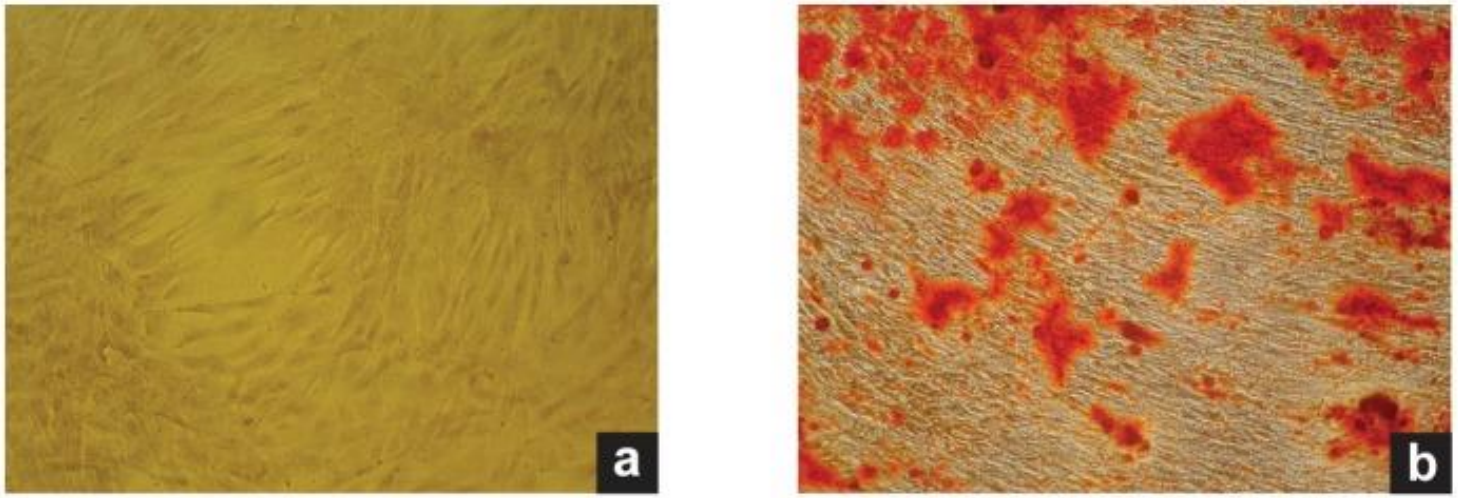


Figure 3. Ad-MSC characterization: A. Osteogenic differentiation of Ad-MSC. Osteogenic differentiation was evidenced by the detection of calcium deposits with Alizarin Red staining.
a. Control Ad-MSCs without osteogenic induction.
b. Ad-MSC cultured for 3 weeks in osteogenic differentiation medium.

in Ad-MSC → mineral deposits were detected after culturing for 3 weeks in a osteogenic differentiation medium but NOT in the control Ad-MSC

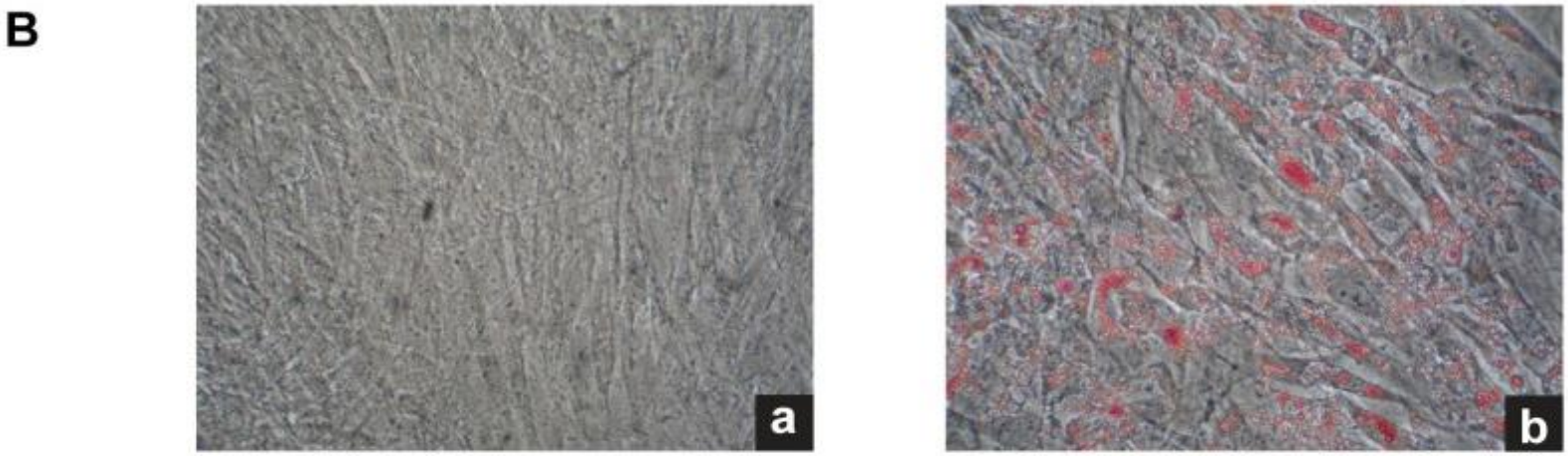


Figure 3. Ad-MSC characterization

B. Adipogenic differentiation of Ad-MSC. Adipogenic differentiation was evidenced by the formation of lipid vacuoles after three weeks of cultivation in adipogenic induction medium.
a Control cells without induction.
b Lipid vacuoles staining with oil red O. 106 magnification.

→ after 3 weeks of treatment with adipogenic induction medium → observing of lipid vacuoles after oil red O staining in Ad-MSC but NOT in control Ad-MSC!

Table 1. Factors involved in bone regeneration secreted by Ad-MSC cultured under normoxic and hypoxic conditions.

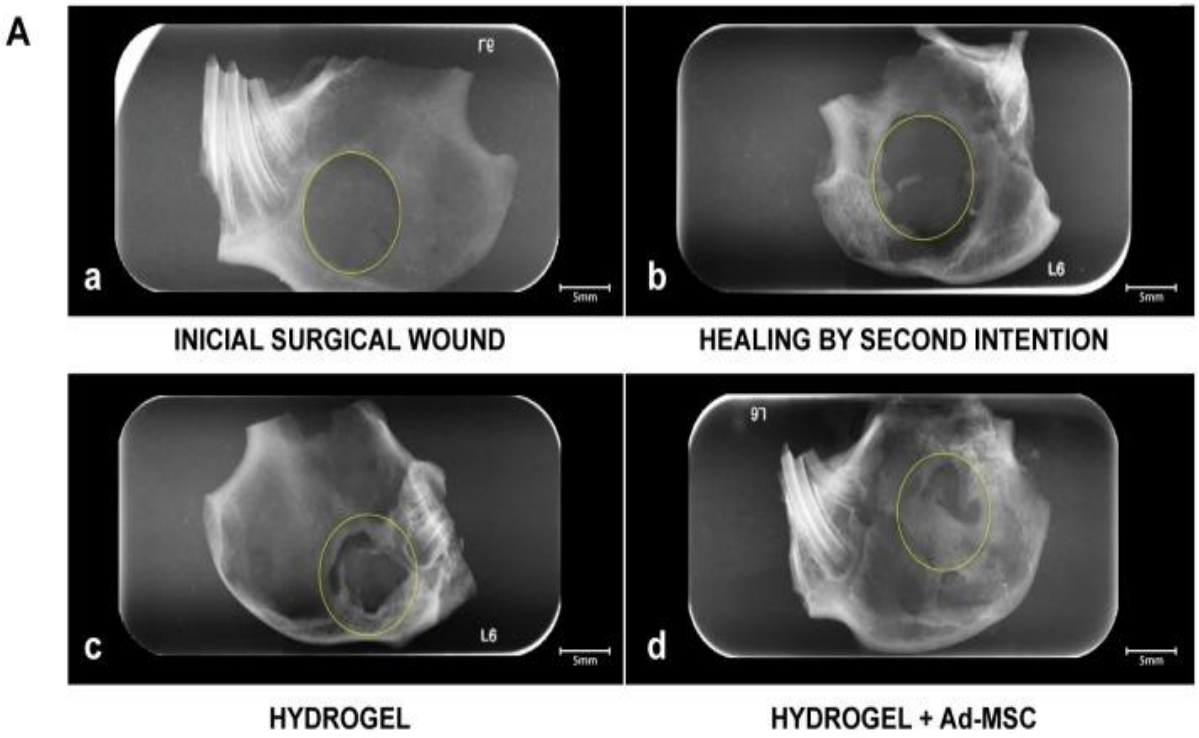
FACTOR	Relative concentration (Arbitrary Units/μg protein)	
	Normoxia	Hypoxia
IL-6	23.8	83.6
VEGF	8.9	19.2
ANGIOGENIN	1.0	7.7
MCP-3	0	11.2
MCP-1	18.9	37.9
IGF-1	5.1	229
TGF - ß	3.7	7.7
PDGF-BB	11.0	23.3
bFGF	10.3	25.9
EGF	7.8	22.7
RANTES	11.2	26.0

The values in the table indicate the relative levels of secretion (Arbitrary Units/μg of protein), of secreted factors produced by Ad-MSC cultured under normoxic and hypoxic conditions. All these factors have been reported to be involved in bone regeneration. IL-6: Interleukin 6, VEGF: Vascular Endotelial Growth Factor, Angiogenin, MCP-3:Monocyte Chemoattractant Protein-3, MCP-1: Monocyte Chemoattractant Protein-1, IGF-1:Insulin Like Growth Factor-1, TGF-ß: Transforming Growth Factor Beta, PDGF-BB: Platelet Derived Growth Factor Isoform BB, bFGF: Basic Fibroblast Growth Factor, EGF: Epidermal Growth Factor, RANTES: Regulated upon Activation Normal T-cell Expressed, and Secreted.

doi:10.1371/journal.pone.0107001.t001

- used Human Angiogenisis Antibody Array C 1000
- showed that Ad-MSC secrete 43 angiogenic factors (cytokines, growth factors, proteases and soluble receptors)
- better secretion in hypoxic conditions compared to cells in normoxic conditions

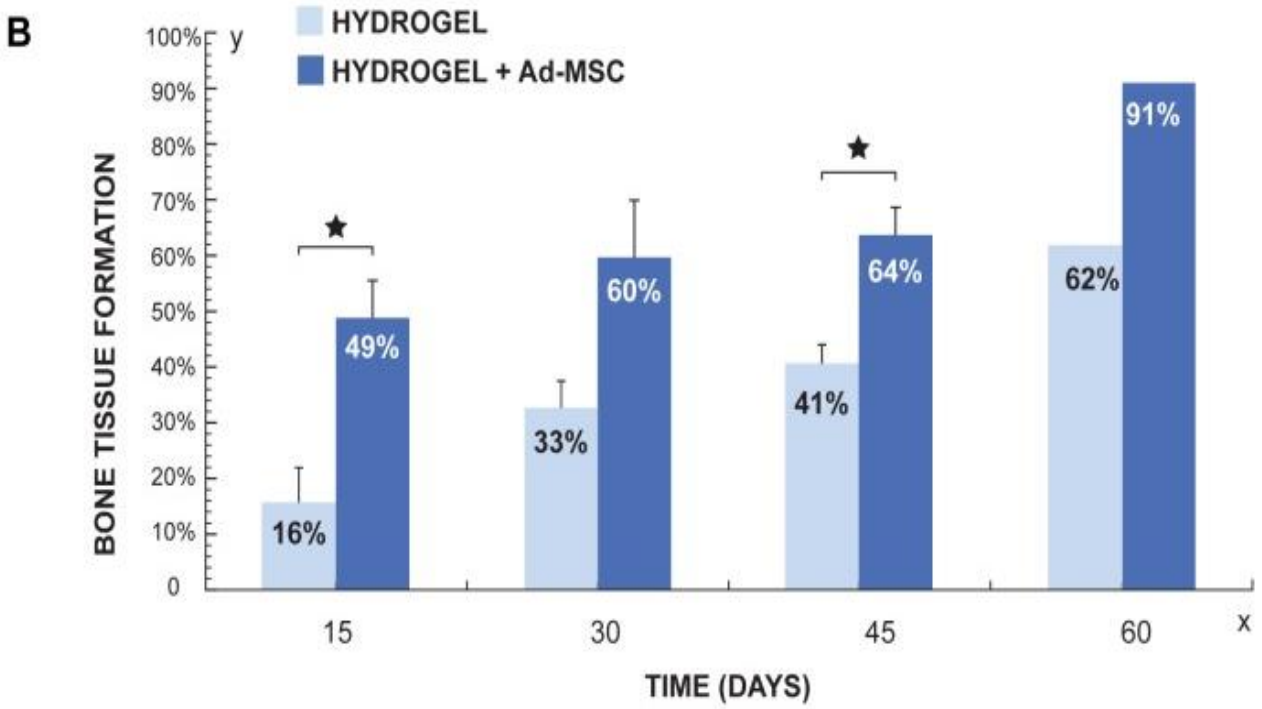
Figure 4. Radiographic Analysis of bone regeneration by implanting HBPHs with Ad-MSC.



A. Radiographic comparison of bone defects at 45 days with different treatments.
a. Initial size of surgical wound.
b. Healing by second intention (without treatment).
c. Bone defect treated with hydrogel.
d. Bone defect treated hydrogel with Ad-MSCs

→ 4Ab: small radiopaque halo from the edges of the bone defects
→ 4Ac: larger radiopaque area from peripheral to the center of the defect after treated with hydrogel without Ad-MSC
→ 4Ad: radiopaque area covering more than 70% of the initial bone lesion after treated with hydrogel with Ad-MSC

Figure 4. Radiographic Analysis of bone regeneration by implanting HBPHs with Ad-MSC.

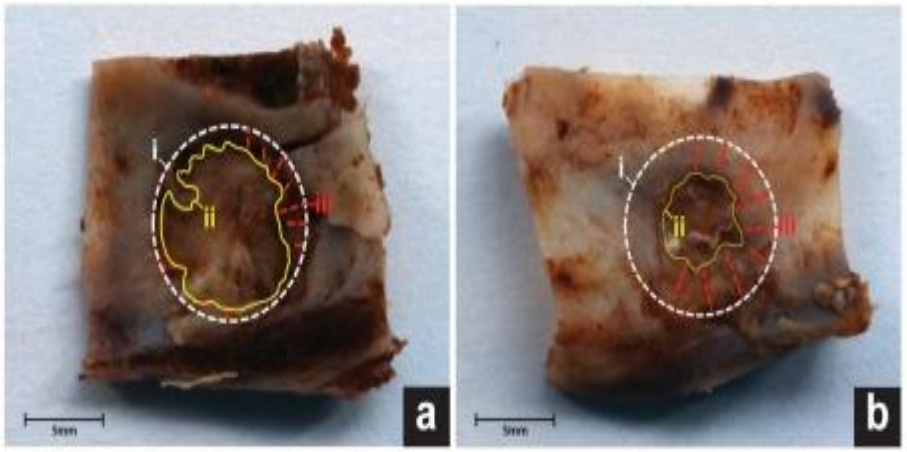


B. Histogram represent the average of newly formed bone tissue at 15, 30, 45 (n = 4) and 60 (n = 1) days after grafting Hydrogel with Ad-MSC (dark blue) and Hydrogel without Ad-MSC (light blue). doi:10.1371/journal.pone.0107001.g004

→more reduction of bone defects in those treated with hydrogel plus Ad-MSC compared to the control side
→60d after surgery nearly 100% closure of the bone defect (performed in a single individual)

Figure 5. Morphometric Analysis of bone regeneration by implanting HBPHs with Ad-MSC

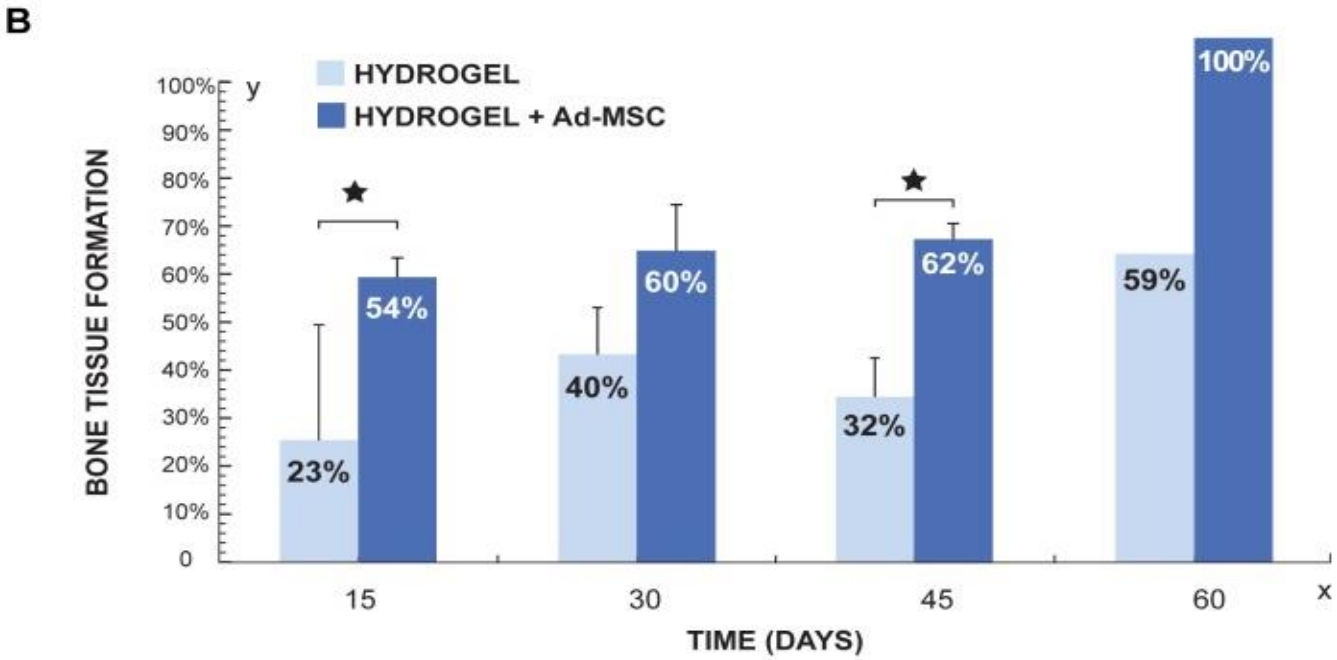
A



A. Surgical specimens 45 days after implantation.
a. Hydrogel.
b. Hydrogel with Ad-MSC.
i, initial bone defect (white circle),
ii, final bone defect (yellow line) and
iii, new formed bone tissue (red lines).

→ more newly formed bone tissue on the side treated with Ad-MSC than the control side

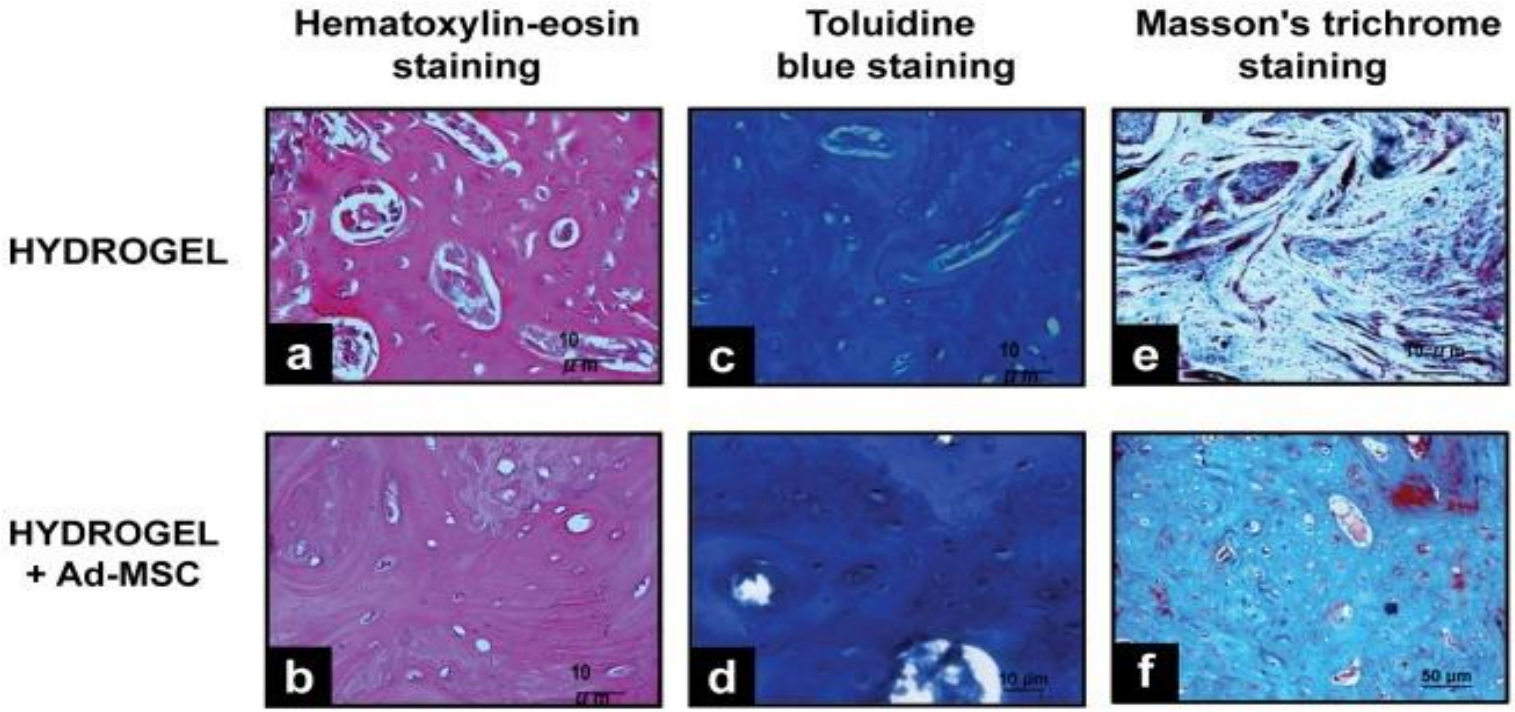
Figure 5. Morphometric Analysis of bone regeneration by implanting HBPHs with Ad-MSC



B. Percentage of newly formed bone at 15, 30, 45 (n = 4) and 60 days (n = 1), after application of Hydrogel with or without Ad-MSC. doi:10.1371/journal.pone.0107001.g005

→significant differences between the treated and control side → more bone formation on the side with Ad-MSC
→in agreement with the results from the radiographic analysis in Figure 4B

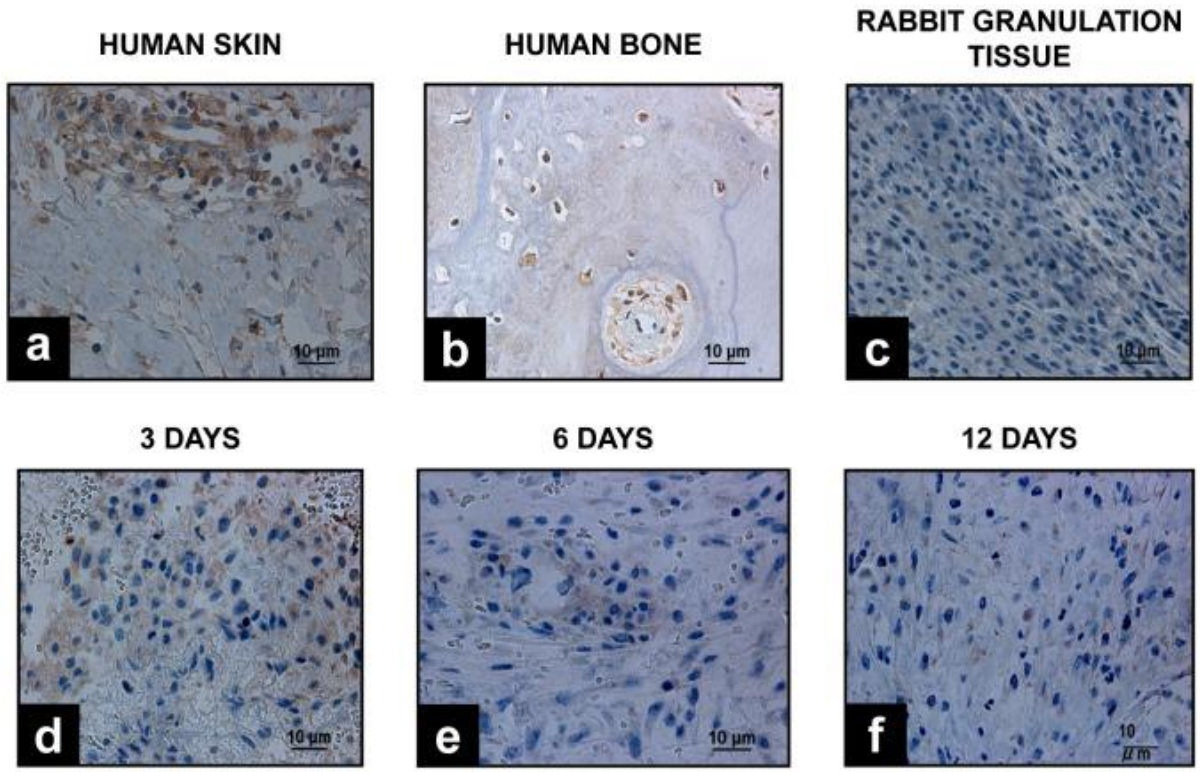
Figure 6. Histological Analysis of bone regeneration by implanting HBPHs with Ad-MSC.



Bone defects treated with Hydrogel and Hydrogel with Ad-MSCs, 45 days after implantation.

→ a, b: hematoxylin and eosin staining, showing a mild chronic inflammatory response.
→ c, d: blue toluidine staining, evidencing intramembranous ossification with little endochondral type ossification
→ e, f: Masson trichrome staining, showing a better organized bone tissue and increased calcification, where hydrogels with Ad-MSC were implanted (Magnification 106).

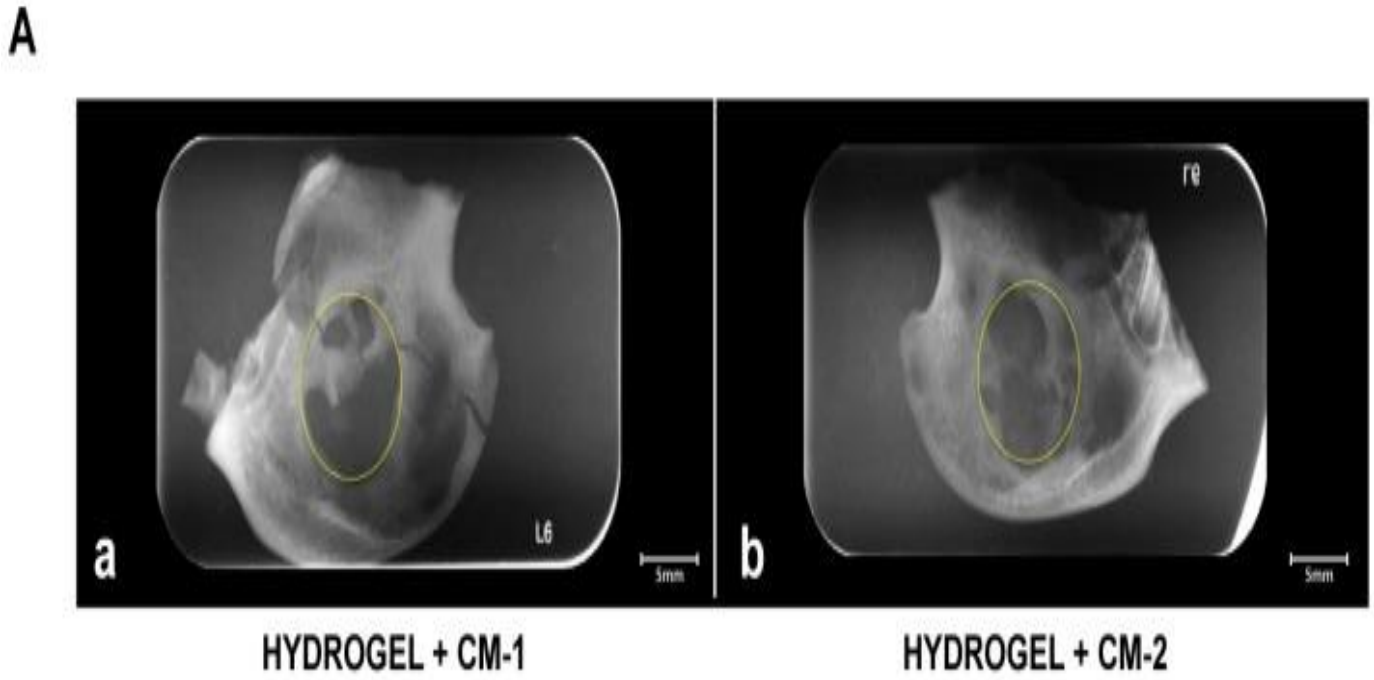
Figure 7. Tracking of implanted Ad-MSC



Immunohistochemical detection of positive human ß-2 microglobulin Ad-MSC.
a: positive control, human skin.
b: positive control, human bone.
c: negative control, rabbit granulation tissue. Tissue regeneration zone after implantation of blood plasma hydrogel with Ad-MSCs.
d: 3 days.
e: 6 days.
f: 12 days

→Ad-MSC remained at the site of the injury during the first 3 days
→6 days after the implantation → reduced cell number
→12 days after implantation → no Ad-MSC detected

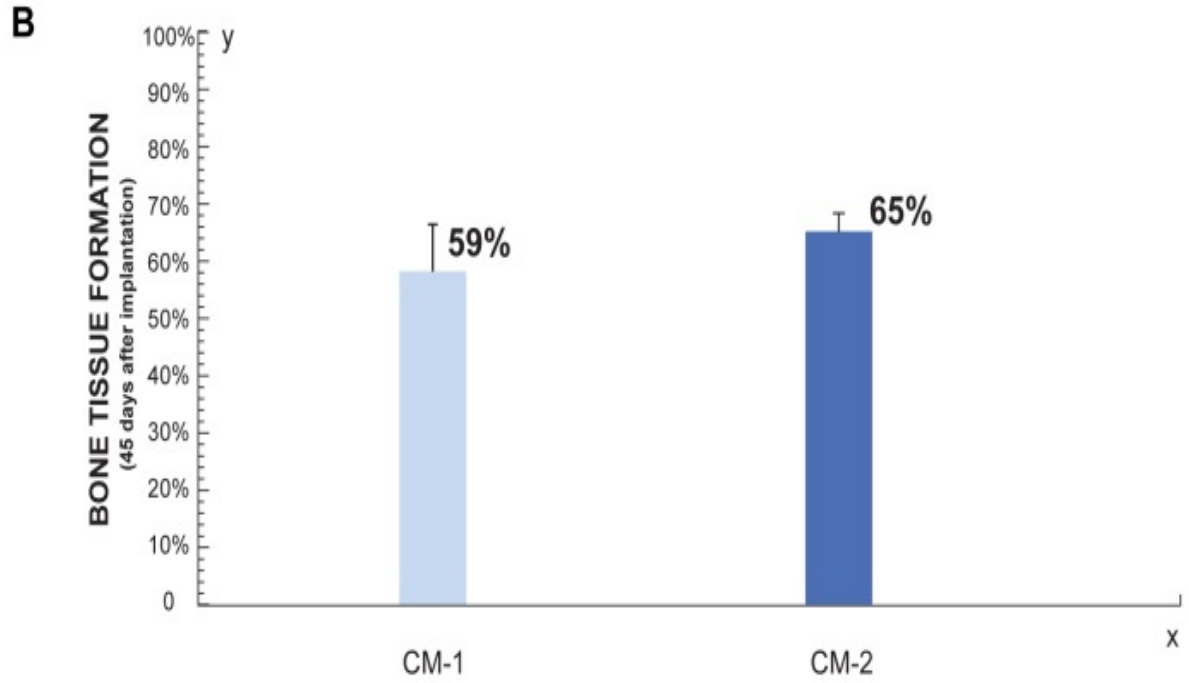
Figure 8. Radiographic Analysis of bone regeneration by implanting HBPHs with CM.



A Radiographic comparison of bone defects at 45 days with CM.
a: Bone defect treated with Hydrogel with CM-1.
b: Bone defect treated with Hydrogel with CM-2.
The circle represents the initial size of bone defect.

→ bone defects implanted with the hydrogel with CM-1 a bridge of well mineralized bone tissue is observed (Figure 8Aa)
→ the defect where hydrogel with CM-2 was implanted →greater amount of newly formed bone tissue but less mineralized (Figure 8Ab)

Figure 8. Radiographic Analysis of bone regeneration by implanting HBPHs with CM.

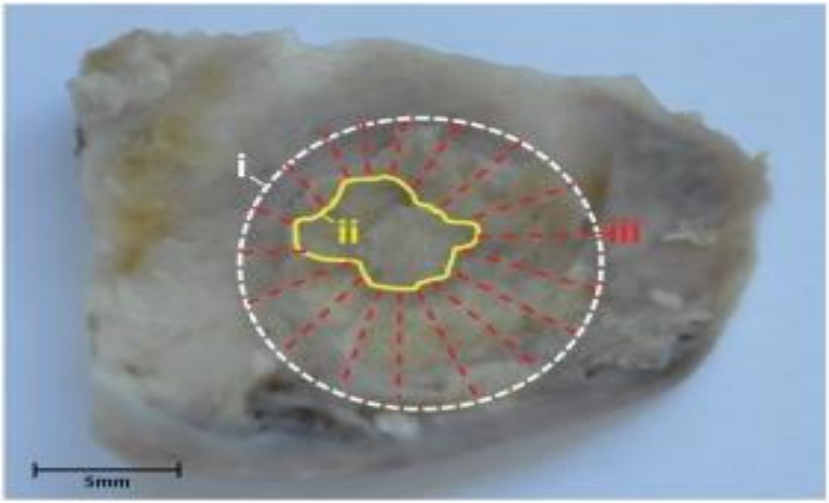


B. Histogram shown the percentage of bone neoformation 45 days after implantation of Hydrogel with CM (n = 3).

→ not statistically significant differences in the amount new bone formed, with the two CM used

Figure 9. Morphometric Analysis of bone regeneration by implanting HBPHs with CM.

A



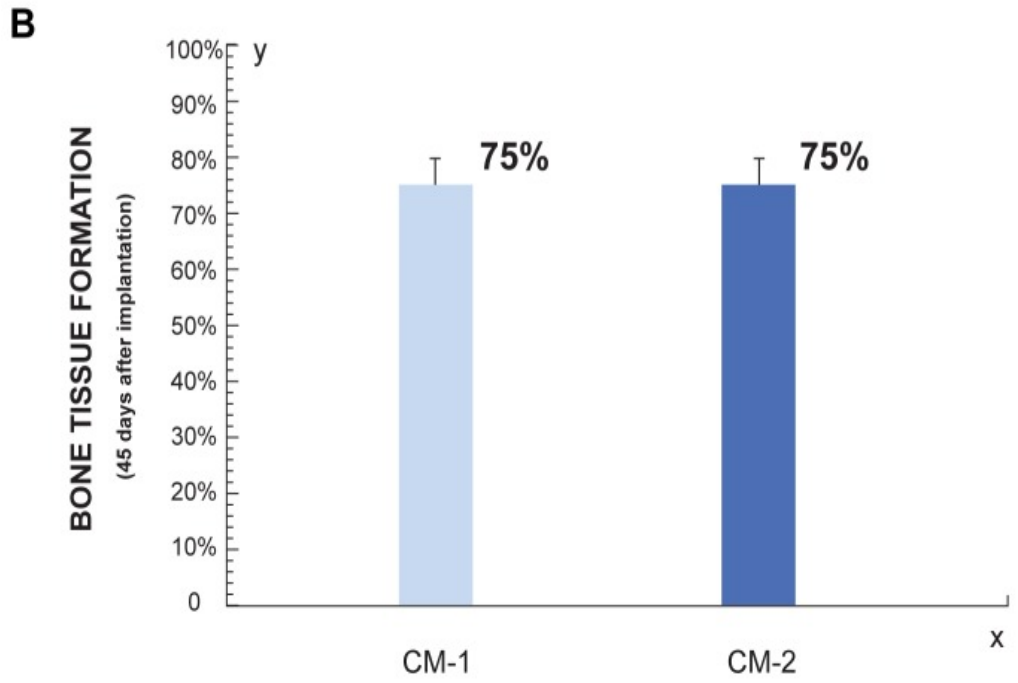
i - Initial bone defect

ii - Final bone defect

iii - Area of bone Tissue Neoformation

→ newly formed bone tissue after 45 d after implantation of hydrogel with CM growing from the periphery to the center of the bone defect

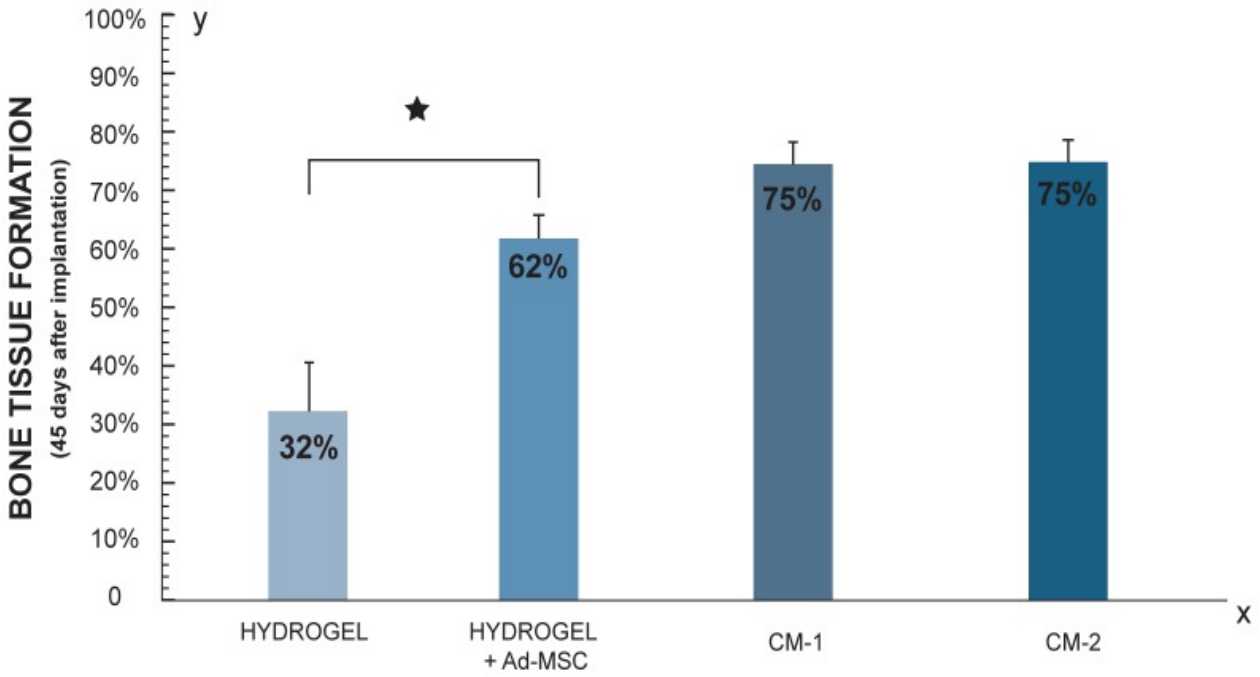
Figure 9. Morphometric Analysis of bone regeneration by implanting HBPHs with CM.



B.
the percentage of bone neoformation 45 days after implantation of Hydrogel with CM (n = 3) is shown.

→ not statistically significant differences between the two protein concentrations of CM used concerning the bone formation

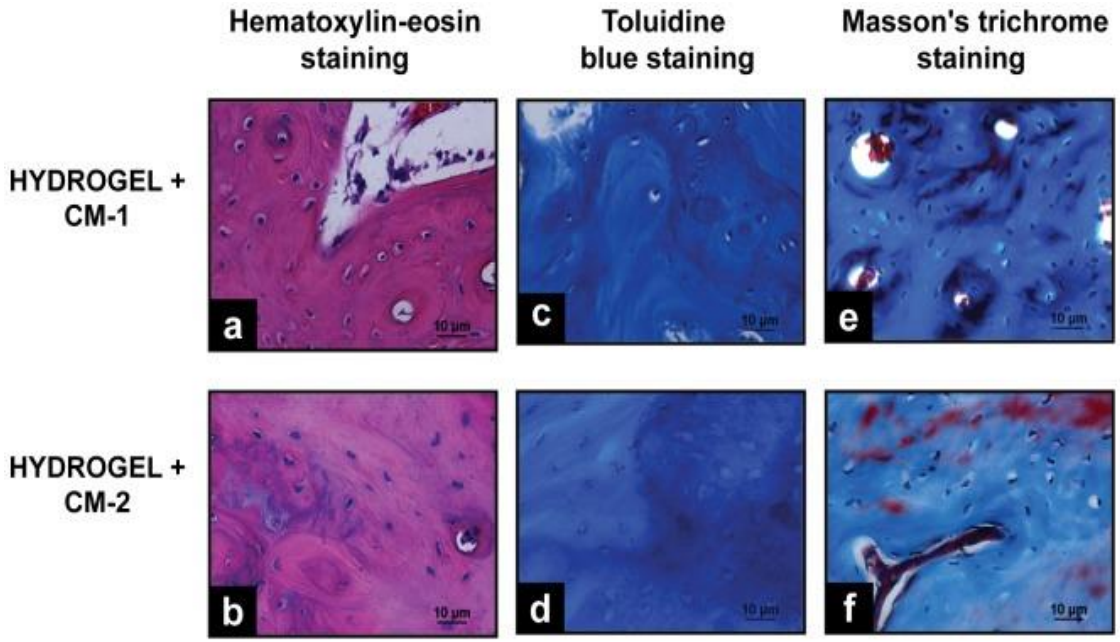
Figure 10. Morphometric Analysis of bone regeneration by implanting HBPHs, HBPHs with Ad- MSC and HBPHs with CM.



Percentage of newly formed bone in defects treated with and without Ad-MSC (n = 4) and CM (n = 3).

→ Bone regeneration improves where hydrogels with Ad-MSC or CM were implanted.
→amount of new formed bone induced by CM was comparable or even higher than that induced by the treatment with Ad-MSC

Figure 11. Histological Analysis of bone regeneration by implanting HBPHs with CM.



Histological Analysis of bone defects treated with Hydrogel and Hydrogel with CM, 45 days after implantation,

→ a, b: hematoxylin and eosin staining → showing a mild chronic inflammatory response. looks more organized than in the control side

→ c, d: blue toluidine staining → evidencing intramembranous ossification.

→ e, f: Masson trichrome staining → showing collagen fibers arranged concentrically around osteoblasts with red small zones → indicating bone mineralization

Discussion

Discussion

- Has the source of MSCs an influence on their effect of bone regeneration capacity?
  - in this study: MSC from adipose tissue
  - But are there better effects of MSC from a different source?
  - MSCs from skeletal muscles have a better effect on bone regeneration than bone-marrow-MSCs (Chatterjea et al.,2010)
- maybe in the future only the condition medium of MSC is used for bone defect
  - the results of this study showed that newly formed bone tissue was comparable or better than the treatment with Ad-MSCs
- MSCs can differentiate to different cell types → other cells are provided → even in the worst case tumor
- different number of animal model used in the study → just 3 rabbits for the treatment with only condition medium and 12 rabbits with the treatment with Ad-MSC → perhaps other results by using equal numbers of rabbits?

Thank you for your attention!

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