Chapter 2

MATERIALS AND METHODS

Materials

1. Materials

1. Agarose, Cat. No. A-9539, Sigma, Germany
2. Athymic mice, HsdCpb:NMRI-nu, Harlan, InterfaunaSüddeutsche Versuchstierfarm GmbH &Co.KG, Germany
3. Cell scraper, Cat. No. 541 070, Greiner, Germany
4. Citrate syringe, 5 ml, Monovetten Gerinnung 5 ml, Cat. No. 05. 1071-026, Sarstedt Monovette, Germany
5. Culture plates, 24 well, Cat. No. 3524, Costar, USA
6. Disposable sterile polystyrene pipette 10 ml, Cat. No. 607 180, Greiner, Germany
7. Disposable sterile polystyrene pipette 25 ml, Cat. No. 760 180, Greiner, Germany
8. Embedding-form, transparent, II/16 mm, Cat. No. 4144, EXAKT, Exakt Appraratebau GmbH, Germany
9. Eppendorf 1.5 ml tube, Cat. No. 0030 120.086, Eppendorf, Germany
10. Falcon culture tubes, 15 ml Polystyrol tube, Falcon, USA
11. Falcon culture tubes, Blue MaxTM 50 ml Polypropylene conical tube, 20x115 mm style, Cat. No. 35 2070, Falcon, USA
12. Falcon tissue culture dish, 100x20 mm style, Cat. No. 35 3003, Falcon, USA
13. Falcon tissue culture dish, 35x10 mm style, Cat. No. 35 3001, Falcon, USA
14. Filter 0.2 µm pore size, Cat. No. FP30/0.2 CA-S, Scheicher & Schuell, Germany

15. Filter paper, 595 S&S Filter paper Circles, 125 mm in diameter, Cat. No. 311 611, Schleicher&Schuell GmbH, German
16. Glass coverslips, 18x18 mm, Knittel Gläser, Germany
17. Glass coverslips, 24x32 mm, Cat. No.19 00 02432, IDL, Exakt Appraratebau GmbH, Germany
18. Glass slide, 76x26 mm/3x1 inch, with frosted end, Knittel Gläser, Germany
19. Glycerin, Cat. No. 4043.1, Roth, Germany
20. Injection syring, 1 ml, Cat. No. 0123, B/Braun Omnifix F, Germany
21. Klinidrape, Sterile, 75x45 cm, Cat. No. 800430-42, Mollycke Health Care, Germany
22. Kodak X-Omat MA Flim, Cat. No. 500 4775, Kodak, Germany
23. Lab-Tech Chamber slides, Cat. No. 177429, Nalge Nunc International, Germany
24. Male Spague Dawley SD rats, 11 month-old, Animal house, Department of experimental medicine, The University of Wuerzburg, Germany
25. Male Spague Dawley SD rats, 2-3 month-old, Harlan, InterfaunaSüddeutsche Versuchstierfarm GmbH &Co.KG, Germany
26. Microscope slides, 50x100x1.5 mm, Cat. No. 41510 EXAKT, Exakt Appraratebau GmbH, Germany
27. Monovettenkanülen Nr2, Cat. No. 85.1162, Sarstedt Monovette, Germany
28. Needle gauge 1, 0.9x40 mm BL/LB 20 G x 1.5′, B/Braun Melsungen AG, Germany
29. Needle gauge 19, 1.10x30 mm BL/LB 19 Gx1.25′, B/Braun Melsungen AG, Germany
30. Needle guage 20, 0.40x20 mm BL/LB 27 G x 3/4", B/Braun Melsungen AG, Germany
31. No. 14: Kapazität 1000µl, 10 Racks á 100 Filter-Tips, Cat. No. 692079, Biozym, Germany
32. No. 2: Kapazität 10µl, 10 Racks á 100 Filter-Tips, Cat. No. 692150, Biozym, Germany
33. No. 8: Kapazität 100µl, 10 Racks á 100 Filter-Tips, Cat. No. no. 692066, Biozym, Germany

34. Parafilm, Laboratory film 4 inches x 125 fts. roll, American National Can, USA
35. PCR softtubes 0.2 ml, Farblos with Deckel, Cat. No. 711000, BioZym, Germany
36. Pipette tip 0.5-10 µl, micro 10 µl, transparent, Cat. No. 771 291, Greiner GmbH, Germany
37. Pipette tip 10 – 200 µl, S type 200 µl, yellow, Cat. No. 739 280, Greiner GmbH, Germany
38. Pipette tip 100 – 1000 µl, Universal, 1000 µl, blue, Cat. No. 740 296, Greiner GmbH, Germany
39. Polishing sand paper for Micro-Scheifsystem EXAKT 400 CS, Grinding sand paper K320, K500, K 1000, K 1200 and polishing sand paper P 2400 and P 4000, Exakt Appraratebau GmbH, Germany
40. Silk 4-0 usp, Cat. No. EH6802H, Ethicon, Germany
41. Sterile disposable injection syringe 1 ml, 26G x 1/2, 0.45x12 mm, B/Braun Melsungen AG, Germany
42. Sterile plastic syringe 5 ml and 2 ml, B/Braun Melsungen AG, Germany
43. Sterile surgical Blade No. 10, B/Braun Aesculap, Aesculap AG &Co,KG, Germany
44. Sterile surgical gloves, Manex ultra, Beiersdorf AG, Germany
45. Vicryl 4-0 usp, Cat. No. V 773 E, Ethicon, Germany

2. Chemicals

1. 1. 1α,25-dihydroxychloecalciferol, Cat: No. D-1530, Sigma, Germany
2. 2-Amino-2-methyl-1-proponol, Cat. No. 08578, Fluka, Germany
3. β-Glycerophosphate, Cat. No. G-9891, Sigma, Germany
4. Absolute ethanol, Cat. No. A1613, Applichem, Germany
5. Accutase, Cat. No. L11-007, PAN, Germany
6. Acetic acid 100%, Cat. No.3738.2, Carl Roth GmbH, Germany
7. Acetone, Cat. No. 8002, J.T.Baker, Germany
8. Acid fuchsin, Cat. No. 1.05231.0025, Merck, Germany
9. AEC Chromogen system, Cat. No. 1477, Immunotech, Germany

10. Albumin, Bovine, Cat. No. A-7906, Sigma, Germany
11. Alcian blue, Cat. No. 05500, Fluka, Germany
12. Alkaline Phosphatase (AP), Leukocyte, Cat. No. 86-C, Blue, Sigma, Germany
13. Aluminium sulphate hydrated, Cat. No. A7523, Sigma, Germany
14. Ascorbic acid, Cat. No. 1.00127, 100 g, Merck, Germany
15. Azophloxin, Cat. No. 11640, Fluka Chemie Ag, Switzerland
16. Benzine cleaning solution, Wund benzin, Cat. No. UN-Nr: 3295, Phamaceutical department, The University hospital, The University of Würzburg, Würzburg, Germany
17. Bovine thrombin, bovine plasma, Cat. No. T-4648, Sigma, Germany
18. Bromphenolblau, Cat. No. 115-39-9, Merck, Germany
19. Calcium chloride, Cat. No 1.02389.100, Merck, Germany
20. Calcium kit, Cat. No. 587M, Sigma diagnostic, Germany
21. Calcium/Phosphate standard set, Cat. No. 360-11, Sigma, Germany
22. Cell Proliferation WST-1 Reagent, Cat. No. 1 644 807, Roche, Germany
23. Chloroform J.T.Baker, Cat. No. 7386,2,5,1 (Phamaceutical department, The University hospital, The University of Würzburg), Mallinckrodt Baker B.V., Netherlands
24. Culture medium DMEM-F12 (1:1), Cat. No. FG 4815, Biochrom KG, Germany
25. DC Protein Assay Kit, Cat. No500-0116, Bio-Rad, Germany
26. De-ionize water, aqua ad injectabilia Delta-Pharma, Pharmarlogical number 6186461.00.00, Boehringer Ingelheim Delta-Pharma GmbH, Germany
27. Dexamethasone, Cat. No. D1756, Sigma, Germany
28. Di-sodium tetraborate anhydrous analytical grade, Cat. No. 106306, Merck, Germany
29. DPBS, Cat. No. P04-36500, PAN, Germany
30. EDTA, Cat. No. EDS, Sigma, Germany
31. Ethanol absolute for analysis, Cat. No. A 1613,1000, AppliChem, Germany
32. Ethydiumbromide, 1%, Cat. No. 2218.2, Carl Roth GmbH, Germany
33. Formaldehyde 37%, Cat. No. K27103803 943, Merck, Germany
34. Fungizone, Amphotericin B 250UGMI, GIBCO Germany

35. Gaunidin HCl (Guanidinehydrochloride), Cat. No. 00037.1, Carl Roth GmbH, Germany
36. Giemsa-Azur-Eosin-Methylene blue solution, Cat. No. 109204, Merck, Germany
37. Glycerol gelatin, Cat. No. GG-1, Sigma diagnostics, Germany
38. Heparin, Calciparin (0.3 ml/7,500 I.E.), Cat. No. BRL, 15290-018, Sanofi Winthrop GmbH, Germany
39. HEPES-Pufferan, Cat. No. 91054, Carl Roth GmbH, Germany
40. Human TGF-β1 immunoassay, Cat. No. DB 100, R&D systems, Germany
41. Hydrochloric acid 37%, Cat. No. 1.00317.1000, Merck , Germany
42. Hydrochloric acid, 1mol/l (1N), Cat. No. UN1789, Merk, Germany
43. Hydrogen peroxide 30 %, H_2O_2, Cat. No. 062262B (Phamaceutical department, The University hospital, The University of Würzburg), Hedinger GmbH, Germany
44. Ketanest, (S) Ketaminhydrochlorid, 5 mg/ml, Cat. No 37086.00.00, Parke-Davis GmbH, Germany
45. Lightgreen, Cat. No. 1.15941.0025, Merck, Germany
46. May-Grünwald, Cat. No. 1.01424.0500, Merck, Germany
47. Methacrylicacid-hydroxyethylester, Cat. No. 28744, PSI Grünewald GmbH, Germany
48. Methanol J.T.Baker, UN1230, Mallinckrodt Baker B.V., Netherlands
49. Methacrylicacid-hydroxyethylester (GMA), Cat. No. 28744, PSI Grünewald GmbH, Germany
50. MicroPoly(A) Pure Kit, Cat. No. 1918, Ambion, UK
51. Molybdatophosphoric acid, Cat. No. 1.00532.0025, Merck, Germany
52. Monoclonal antibody for Collagen type I, Cat. No. C2456, Sigma, Germany
53. Monoclonal antibody to Bovine osteocalcin, Cat. No. M 041, Takara Shuzo Co., LTD., Germany
54. Neutral red solution 0.5% buffered , Cat. No. N 6264, Sigma, Germany
55. Nuclear Fast Red, 5 g, Cat. No. 8002, Sigma, Germany
56. Oil Red O, 25 g, Cat. No. 75087, Fluka, Germany
57. Orange-G, Cat. No. 1.15925.0025, Merck, Germany

58. Penicillin/Streptomycin 10,000U Penicillin/ml and 10 mg Streptomycin/ml sterile filtered, Cat. No. P06-07100, PAN, Germany
59. p-Nitrophenol, 5g, Cat. No. 104-8, Sigma, Germany
60. p-Nitrophenylphosphate disodium salt, Cat. No. 1.06850, Merck, Germany
61. Poly-D-Lysine, Cat. No. 644 587, Sigma, Germany
62. Ponceau Xylidin, Cat. No. 11640, Fluka, Switzerland
63. Precision adhesive, Heraeus Kulzer GmbH, Germany
64. Propyleneglycol, Cat. No. 16033, Fluka, Germany
65. Pyronin G, Cat. No. 7518, Merck, Germany
66. Recombinant bone morphogenetic protein – 2 (rhBMP-2), Department of Physiochemistry, The University of Wuerzburg, Wuerzburg, Germany
67. Rnase Inhibitor, Cat. No. 799017, Roche, Germany
68. Rompun 2%, Xylazinhydrochlorid, for animals, bovine, horse, dog and cat, 25 ml, Cat. No. PZN-1320422, BAYER, Germany
69. R.T.U. Vectastain Universal Elitee ABC peroxidase kit, Cat. No. PK 7200, Vector Laboratories, Inc., UK.
70. Set of 100mM dATP, 100mM dCTP, 100mM dGTP, 100mM dTTP, Deoxynucleoside triphosphate set, Cat. No. 1 277 049, Roche, Germany
71. Silver nitrate, Cat. No. 1.01512.0025, Merck, Germany
72. Sodium Chloride, Cat. No. S-9888, Sigma, Germany
73. Sodium hydrogenphosphate heptahydrate (dibasic), Cat. No. 22,199-6, Aldrich Chemical Company Inc., USA.
74. Sodium hydrogenphosphate, monobasic monohydrate. Cat. No. 22,352-2, Aldrich Chemical Company Inc., USA.
75. Sodium hydroxide pellets, Cat. No. 1 06469, Merck, Germany
76. Sodium hydroxide solution 1 M, Cat. No. 50049671, Merck, Germany
77. Sodiumazid 99%, Cat. No. 19,993-1, Sigma, Germany
78. Superladder-mid1 100bp ladder, Cat. No. SLM-100, Carl Roth GmbH, Germany
79. Technovit 7200 VLC, Heraeus Kulzer GmbH, Germany
80. Technovit light hardening adhesive, Heraeus Kulzer GmbH, Germany

81. Titan™ One Tube RT-PCR system, 100 reactions, Cat. No. 1855 4f76, Roche, Germany
82. Toluidine blue (O-Certified) 85%, Cat. No. T0394, Sigma, USA
83. Tris(Tris-(hydroxymethyl)-aminomethane), 500g, Cat. No. 5429.1, Carl Roth GmbH, Germany
84. Trypsin/EDTA solution (10x) 0.5%/0.2% (w/v), Cat. No. L 2153, Biochrom KG, Germany
85. Weigert Isenhämatoxylin, Solutions A and B, Cat. No. 1.15973./1 and 1.15973/2, Merck, Germany
86. Xylencyanol for electrophoresis, Cat. No. 2650-17-1, Merck, Germany

3. Equipment and instrument

1. Animal house for rats and immunodeficient mice, Experimental medicine department, Dental school, The University of Wuerzburg, Germany
2. Balance, Waage JL-180, Wägebereich 180 g, Auflösung 0.001 g, Kern Wägebereich, Germany
3. Balance, Waage MK-500 C, Wägebereich 500 g, Auflösung 0.1 g, Kern Wägebereich, Germany
4. BioRad Power supply, model 1000/500, BioRad, Germay
5. BioRad-electrophoresis light machine, model Gel Doc 2000, BioRad, Germay
6. Blood cell counter machine, Coulter MD Serie, Beckman Coulter GmbH, Germany
7. Bone cutting saw 10620, Bizerba, Germany
8. Centrifugator, Biofuge 13, Heraeus Instruments; Germany
9. Centrifugator, Jouan BR4i, Jouan GmbH, Germany
10. Centrifugator, Universal 30 - RF Nr. 1402, Hettich-Zentrifugen, Germany
11. Coulter cell counter machine, Casy 1, Cell counter-Analyser System Model TTC, Schäfe system GmbH, Germany
12. Culture incubator, B 5060 EK/CO2, Heraeus-Instruments, Germany
13. ELISA –Tecan Rainbow Spektralphotometer-Spectronic 301, Milton Roy, Germany
14. Exakt-cutting-grinding system, PSI, EXAKT, Grünewald GmbH, Germany

15. Gel analysis softwear, BioRad-Multi-Analyst, Bio Rad, Germany
16. Homogenisator, modified for collagen matrix cutting with cylindrical shaped cutter, size 5 mm in diameter, Framo LR20, Franz Morat KG GmbH&C, Germany
17. Hot stream evaporator, Franz Morat KG GmbH&C, Germany
18. Household refrigerator, Bosch, Germany
19. Laminar air work bench, Heraeus Lamin Air TL 2448, Heraeus-Instruments, Germany
20. Light microscope, Umkehrmikroskop CK 2/SC35/12, Olympus Optical, Germany
21. Lyophilisator, Beta 1-8, LDC-2, Martin Christ, Germany
22. Magnetic stirring machine, Model - M23 180 mm Durchmesser, Hartenstein, Germany
23. Micro-pipette, Eppendorf research, 10, 20, 100, 200, and 1000 µl, Research 3110 Pipetten, Eppendorf, Germany
24. Microscope, Mikroskop Leitz DM RBE, Leica Ismaning, Germany
25. Microwave oven, Sharp-Carousel, Sharp, Japan
26. Minus 70°C refrigerator, Cat. No. ECC 6085-5, Tiefkältetruhe ProfiLine - Bader Kältetechnik, Germany
27. Mitsubishi video printer,Model P90, Mitsubishi, Japan
28. One dimensional gel running tray, Model B2, Owl Separation systems. Inc, USA
29. Packages of sterile trays, cotton pliers, bone cutter, and minor surgery set, Cranio-maxillofacial surgery unit, The University of Wuerzburg, Germany
30. PCR Thermocycle machine, PCR express Hybrid, Hybrid, Germany
31. pH Meter, pH 523, Wissenschaftlich-technische Werkstätten GmbH, Germany
32. Shaker machine, Desaga, Cat. No. DE 24, Sarstedt-Gruppe, Germany
33. Thermomixer 5436, Eppendorf, Germany
34. Vortexter, Model VF2, Janke-Kunkel, Germany
35. Warm water bath, Schüttel-Wasserbad 3047, Köttermann GmbH, Germany
36. X-ray machine, as use in x-ray clinic
37. X-ray machine, Faxitron 43855/B, Hewlett-Packard, USA

4. Software

1. Gel analysis software, BioRad-Multi-Analyst, BioRad, Germany
2. The public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/)
3. Stat View for windows, version 5, SAS Institute Inc, USA.

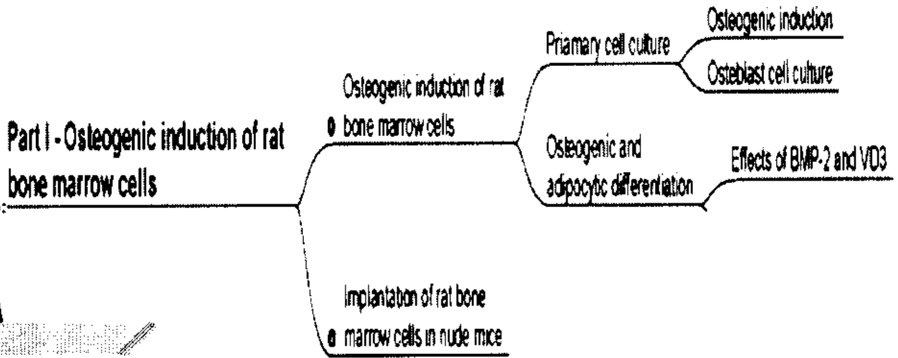
Methods

The study comprises of two parts; Part I: Osteogenic induction of rat bone marrow cells and, Part II: Studying effects of PRP on growth and differentiation of premature osteoblasts and mesenchymal stem cells in rat bone marrow (Figure 2- 1).

1. Scope of the study

- 1.1. Osteogenic induction of rat bone marrow cells
    - 1.1.1. Effects VD3 and rhBMP-2 in rat bone marrow cell culture
    - 1.1.2. An implantation of rat bone marrow in athymic mice
- 1.2. Effects of platelet-rich plasma (PRP) on growth and osteogenic differentiation of rat bone marrow
    - 1.2.1. Effects of PRP in three-dimensional cell culture, *in vitro* study
    - 1.2.2. An implantation of bone marrow and PRP in immunodeficient mice, *in vivo* study
- 1.3. Measurement of TGF-β1 content in PRP

Figure 2- 1 Scope of the study



Scope of Study

Part II -Effects of PRP on proliferation and differentiation of rat bone marrow cells

in vitro
3-D cell culture of differentiated cells

in vivo
Intra-muscular implantaion of fresh bone marrow

2. Study designs

2.1. Osteogenic induction of rat bone marrow cells

Bone marrow was harvested from femurs and tibias of Spraque-Dawley (SD) rats. In primary passage, 1/6 or 16.7% of fresh bone marrow from one rat was seeded in a 35 mm culture plate with a surface area of 9.6 cm^2. At the initial confluent or around the 13th -14th culture-days bone marrow was subcultivated (For detail procedure see section 3.1.1). Cells were seeded in a concentration of $5x10^3$ cells/cm^2 or $5x10^4$ cells/ 35 mm plate in first passage (For detail procedure of cell seeding see section 3.2.1).

Bone marrow was cultivated in mineralized medium supplemented with 20 nM dexamethasone in 5% CO_2 at 37°C for a period of 21 days in the primary and first passages. rhBMP-2, VD3 and TGF-β2 were added in the culture medium as they were stated in the study design of each group.

Adipocytic, chondroblastic and osteoblastic differentiations were investigated. Oil red O and Alcian blue stainings were performed to identify adipocytes and chondroblasts, respectively. Alkaline phosphatase activity (ALP), expression of type I collagen and osteocalcin in extracellular matrix, expressions of ALP, type I collagen, BMP-2, and osteocalcin mRNAs and *in vitro* mineralization were detected as markers of osteoblastic differentiation. The investigations were performed in doublet or triplet and average values were used in the analysis (For detail procedures to characterize type and growth of differentiated cells see section 3.3).

2.1.1. Effects of 1α,25 dihydroxycholecalciferol (VD3) and rhBMP-2 in rat bone marrow cell culture

Bone marrow was harvested from femurs of 30 of the 10 – 11 month-old Spraque-Dawley rats. In Group A, bone marrow cells were cultivated in mineralized culture medium supplemented with 20 nM dexamethasone for the whole period of cell culture. In Group B, at 30% confluence or around the 4th -5th culture-day of primary and first passages, 50 ng/ml rhBMP-2 was added in the mineralized culture medium supplemented with 20 nM dexamethasone. In Group C, at 70% confluence or around 8-9 culture-day, 10 nM VD3 was added in the mineralized culture medium without

supplementation of dexamethasone. Expressions of ALP, type I collagen, BMP-2 and osteocalcin mRNAs were detected on the 7th, 14th, and 21st culture-days and *in vitro* mineralization was detected on the 21st culture-day of each passage. Groups of study are demonstrated in Table 2- 1.

Table 2- 1 Effects of VD3 and rhBMP-2 in rat bone marrow cell culture

Bone marrow cell culture		**Initial cell seeding – at 30% confluence**	**At 30% - 70% confluence**	**70% confluence – 21 culture-day**
Control group	**Group A**	Mineralized medium + 20 nM Dexamethasone		
Experimental groups	**Group B**	Mineralized medium + 20 nM Dexamethasone		Mineralized medium *without* Dexamethasone + **10 nM VD3**
	Group C	Mineralized medium + 20nM Dexamethasone	Mineralized medium +Dexamethasone + **50 ng/ml rhBMP-2**	

2.1.2. An implantation of differentiated bone marrow cells and fresh bone marrow in immunodeficient mice

2.1.2.1. Bone marrow cells harvesting and culturing

Bone marrow was harvested from femurs of 10 of the 2-3 month-old Spraque-Dawley rats. It was cultivated in a mineralized medium with 20 nM Dexamethasone and 10 nM VD3 until cell culture reached confluence in the first passage. Cultivated bone marrow cells, $1x10^5$ cells, in the first passage or 1/5 of the fresh bone marrow from one rat were seeded on 3x5 mm ICBM scaffold (Insoluble collagenous bone matrix). Bone marrow cells were incubated in a mineralized medium supplemented with 20 nM dexamethasone in 5% CO_2 at 37°C for 12 hours, before they were implanted in immunodeficient mice (nude mice).

2.1.2.2. Implantation and groups of study

The ICBM scaffolds were implanted in right and left subcutaneous pockets on the dorsal side of the nude mice, one scaffold in one pocket. There are three groups in

the study, Group A: ICBM scaffold and fresh bone marrow, Group B: ICBM scaffold and cultivated bone marrow cells, and Group C: ICBM scaffold without cells. A total of 22 ICBM scaffolds were subcutaneously implanted in 12 nude mice. The groups of study are demonstrated in Table 2-2.

2.1.2.3. Explantation and an evaluation of bone formation

Mice were sacrified at 6, 18, 28 and 45 days after implantation. Explants were immediately radiographed using Faxitron at 25 kV and 35 sec. Decalcified specimens were embedded in paraffin and stained with Giemsa and Masson Trichrome stainings. Undecalcified specimens were embedded in Technovit 7200 VLC and double stained with Giemsa and von Kossa staining. The histological pattern and progress of new bone formation were observed under light microscope (For staining of histological specimen seeing section 3.5.2).

Table 2- 2 An implantation of bone marrow cells in nude mice

Groups	Characteristics	Numbers of ICBM / time point	Sacrification times	Numbers of ICBM	Numbers of mice
A	ICBM scaffold with Fresh bone marrow	2	At 18, and 45 days	4	2
B	ICBM scaffold with Bone marrow cells in 1st passage	4	At 6, 18, 28, 45 days	16	8
C	ICBM scaffold without cell	1	At 28 and 45 days only	2	2
	Total			22 scaffolds	12 mice

2.2. Effects of PRP on growth and osteogenic differentiation of rat bone marrow

2.2.1. Effects of PRP in three-dimensional cell culture, an *in vitro* study

PRP and PPP were added on a three-dimensional cell culture of differentiated bone marrow. Growth and differentiation of cells were monitored. The effects were compared with the effects of rhBMP-2. The study design is demonstrated in Figure 2-2.

2.2.1.1. Groups of study

Groups of study were categorised into three main groups, (I) experimental, (II) positive control, and (III) negative control groups. In experimental groups, various numbers of platelets were added on each scaffold. to study theirs dose dependent effects (Groups A-C). The effects of PRP in experimental groups were compared with the effects in positive control groups, platelet poor plasma (PPP) (Group D), 300 ng lyophilised rhBMP-2 (Group E) and 20 nM dexamethasone only (Group F) . Possible interferences from blood products and mineralization of the structure of ICBM were monitored in negative control groups (Groups G-I) (Cell seeding on ICBM scaffold) (Table 2- 3).

Bone marrow was harvested from femurs of 50 of the two-month-old male Sprague Dawly rats (SD rats), weighting 280 – 300 grams. Bone marrow cells on the 7th-8th culture-days in the primary passage were pooled and, 4×10^4 cells were seeded on each 3x5 mm ICBM scaffold (See detail procedures of cell seeding in section 3.2.2.1). Culture of bone marrow cells in the primary passage and in three-dimensional cell culture were cultivated in a mineralized medium supplemented with 10% FBS and 20 nM dexamethasone in 5% CO_2 at 37°C through-out the experiment.

2.2.1.2. Procurement and characterization of PRP product

A total of three hundred and sixty-three millilitres of whole blood was drawn from 41 of the 2 month-old male SD rats. Concentrations of platelets and white (WBC) and red blood cells (RBC) from PRP, PPP and whole blood were counted.

Blood products were smear on glass slides and stained with May-Gruenwald and Giemsa staining (For detail procedures of PRP preparation and characterization see sections 3.4.1 and 3.4.2).

2.2.1.3. An addition of PRP and PPP on ICBM scaffolds

A. Numbers of platelets and amount of PPP applied on each scaffold

PRP containing 2.5×10^8 platelets (38 μl – 40 μl) was added on each scaffold in Group A (100% PRP) and Group G (PRP control group). In Groups B (25% PRP), 0.625×10^8 platelets (9.5 μl), and Group C (6.25% PRP), 0.16×10^8 platelets (2.5 μl), were added on each scaffold of each group. Forty microlitres of PPP were added on each scaffold of Group D (cells and PPP), Group E (cells and rhBMP-2) and Group H (scaffold and PPP) (Table 2- 3).

B. Platelet activation

To initiate blood clotting, 10 μl of 1000 unit bovine thrombin in 10% Calcium chloride solution was used to clot 38 - 40 μl of PRP, PPP and whole blood. Five microlitres of the solution was used to clot 9.5 – 10.0 μl and 2.5 μl of PRP. The thrombin-calcium chloride solution was pipeted on each scaffold followed immediately by an adding of blood products. Blood products were clotted with one minute after they were contacted with the thrombin-calcium chloride solution on the scaffolds forming a clotting gel covering the external surface of the ICBM scaffold (Figure 2- 3 and Figure 2- 4).

C. Adding sequence of PRP and PPP

In each set of experiment, blood products were added sequentially on ICBM scaffolds as follow (1) PRP100% (Group A), (2) PRP25% (Group B), (3) PRP 6.25% (Group C), (4) BMP-2 (Group E), (5) PPP groups (Group D), (6) control PRP group (Group G), and (7) control PPP group (Group H).

Approximately 30 minutes after clotting, 1.5 ml of culture medium were added in each well of the 24 well plate in a similar order as the sequence of the adding sequence of PRP and PPP in the previous paragraph, which were Groups A, B, C, E, D, F, G, and H, respectively. Cells were seeded on scaffolds for 4-5 hours before the medium was added in each well.

2.2.1.4. Investigated parameters

WST assay was performed to determine numbers of viable cells. ALP activity and calcium content were measured as parameters of osteogenic differentiation. Neutral red vital stain and ALP staining were carried out to demonstrate attachment, proliferation and differentiation of cells on the structure of the ICBM scaffold (For detail procedures see section 3.3.43.3.4).

2.2.1.5. Investigation times

WST assay and ALP activity were measured every 3 day after cell seeding. The calcium content was measured on the 18th and 21st culture-days. Morphological staining of the neural red vital stain and ALP staining were performed every 6 days after cell seeding.

2.2.1.6. Numbers of samples

ALP activity, calcium content and WST were measured from five samples and morphological staining was stained on three samples at each time point. Measurement of numbers of cells and calcium content were performed on the same sample. Numbers of cells were determined first then it was followed by measurement of the calcium c ontent. In m orphological staining, neutral red vital stain and ALP staining were also performed on the same samples. The ICBM scaffolds were stained with neutral red staining followed by ALP staining.

Eighty-two ICBM scaffolds with cells were investigated in each group (6 groups). A total of 2 x 10^8 cells were seeded on 492 of the 3x5 mm ICBM scaffolds (from 6 groups). Two hundred and ten ICBM scaffolds were used in negative control groups (Table 2- 4).

2.2.1.7. Experimental sequences

The experiment was performed in 2 days. On the first day, samples for WST and calcium content assays and morphological staining were performed. Samples for ALP activity measurement were performed on the second day.

Table 2- 3 Groups of study in the study of effects of PRP *in vitro*

Groups	Characteristics	Volume of PRP or PPP	Volume of clotting solution.
Experimental groups			
A	Scaffold+cells+100%PRP ($2.5x10^8$THR/scaffold)	PRP 38-40 μl	10 μl
B	Scaffold+cells+25%PRP ($0.625x10^8$THR/scaffold)	PRP 9.5 μl	5 μl
C	Scaffold+cells+6.25%PRP ($0.16x10^8$THR/scaffold)	PRP 2.5 μl	5 μl
Positive control groups			
D	Scaffold+ cells +PPP	PPP 40 μl	10 μl
E	Scaffold+cells+300 ng rhBMP-2	PPP 40 μl	10 μl
F	Scaffold+cells	-	-
Negative control group			
G	Scaffold with PRP	PRP 40 μl	10 μl
H	Scaffold with PPP	PPP 40 μl	10 μl
I	Scaffold only	-	-

Table 2- 4 Investigated parameters and numbers of ICBM scaffolds with cells in each group

Parameters	Investigation times (culture-day)	Numbers of samples / time point	Numbers of scaffolds
ALP activity	3, 6, 9, 12, 15, 18, & 21	5	35
WST	3, 6, 9, 12, 15, 18, & 21	5	35
Calcium content	18, & 21	5	-
Morphology	3, 9, 15, & 21	3	12
Numbers of scaffold with cells in each group during a culture period of 21 day			**82**

Figure 2- 2: Study design of the study of effects of PRP *in vitro*

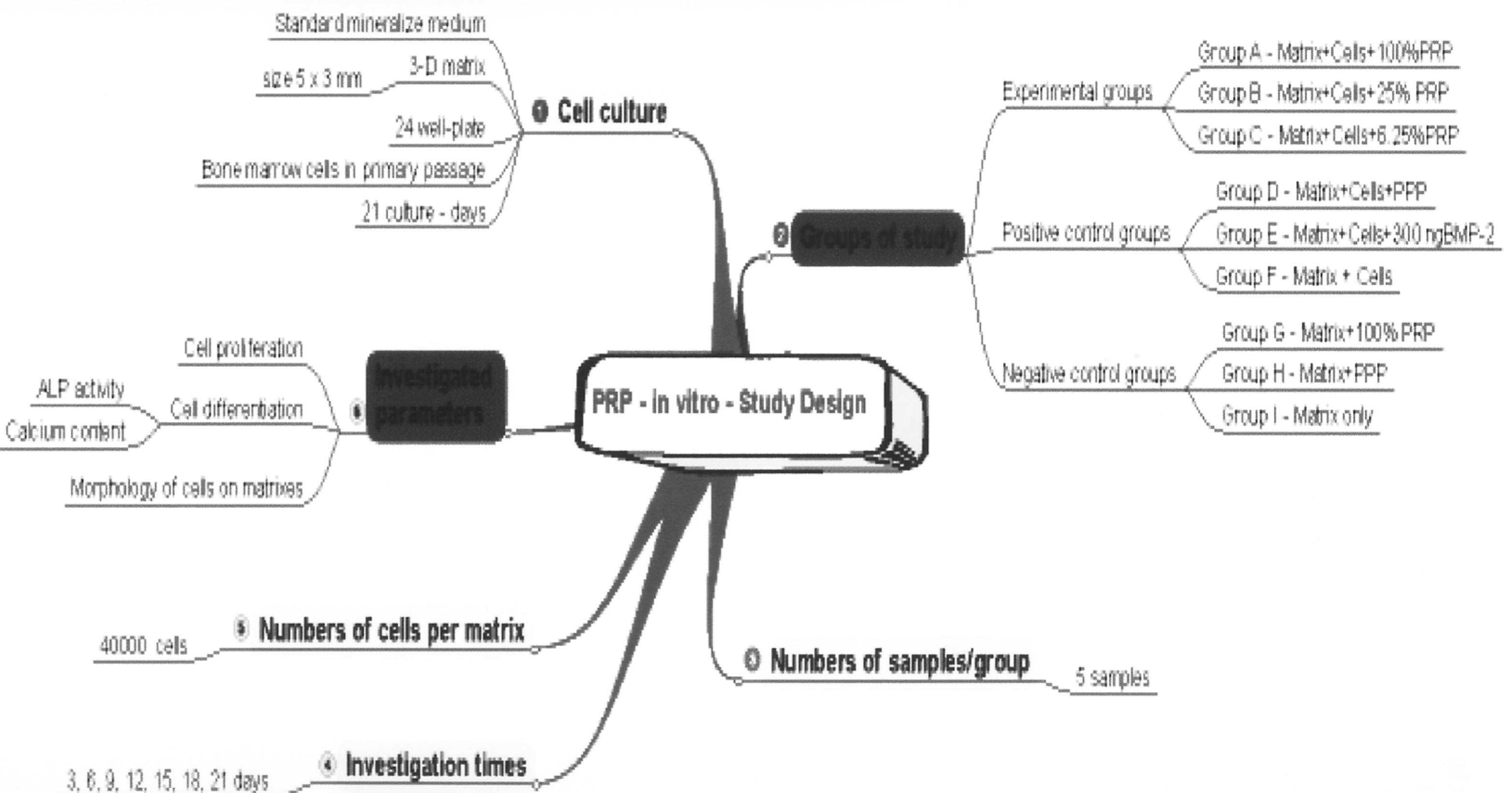


Figure 2- 3 Overview of an addition of PRP in three-dimensional cell culture

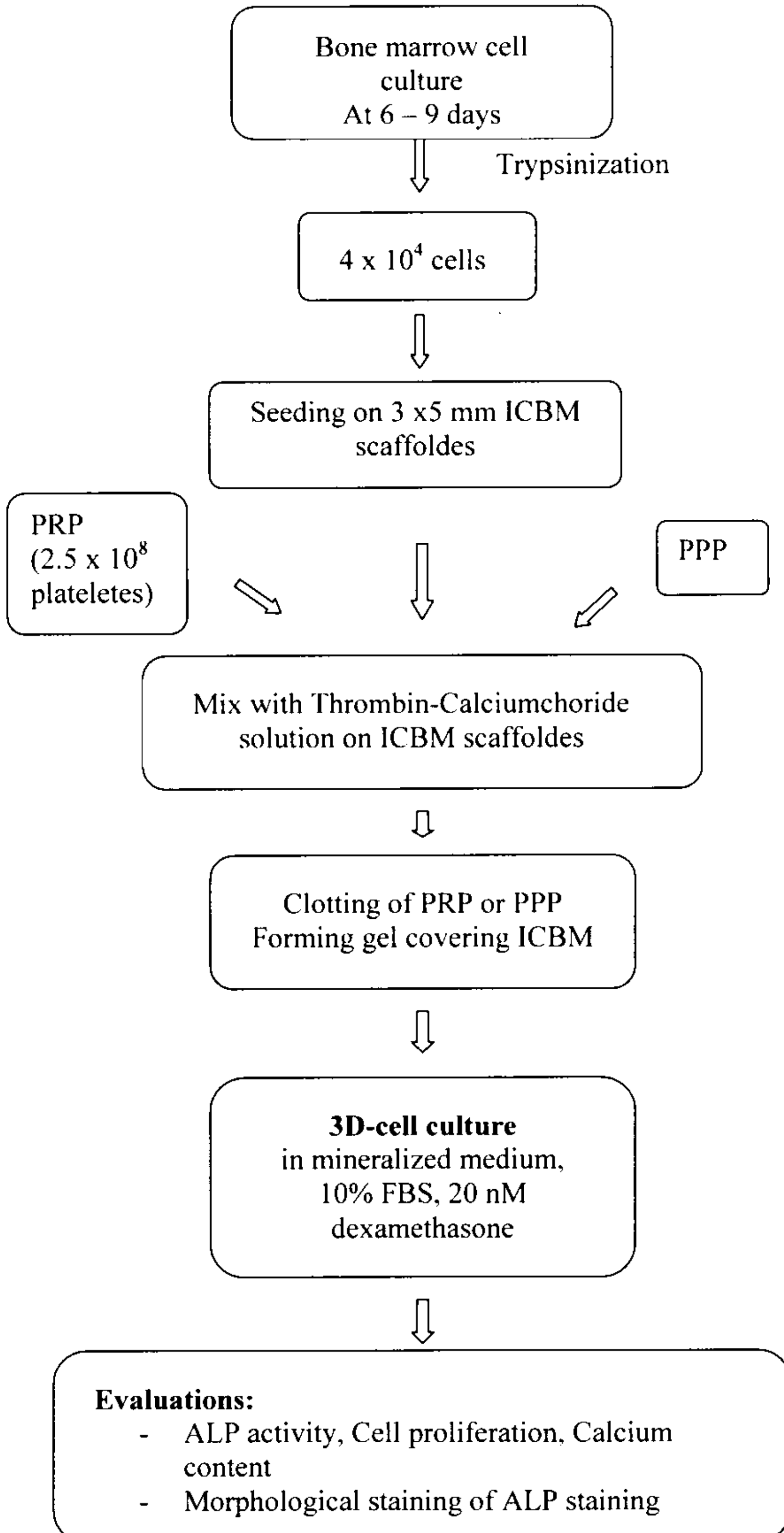
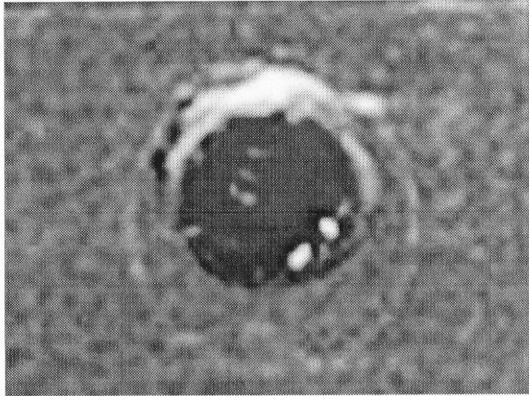


Figure 2- 4 Demonstrating blood clotting on 3x5 mm ICBM scaffold



2.2.2. An implantation of bone marrow in nude mice, an *in vivo* study

The effect of PRP to osteogenic induction of bone marrow *in vivo* was evaluated and compared with the induction effects of whole blood, PPP and rhBMP-2. Fresh bone marrow was mixed with blood products or rhBMP-2 on 5 x 10 mm ICBM scaffolds. The scaffolds were implanted intramuscularly in nude mice. The amount and pattern of the bone formation were evaluated from radiographs and the histology of implanted specimens. The study design is demonstrated in Figure 2- 1 .

2.2.2.1. Groups of study

Groups of study were categorized into 4 main groups; Group I: ICBM scaffold with bone marrow and blood products (PRP, PPP and whole blood) (Groups A-C), Group II: ICBM scaffold with bone marrow and rhBMP-2 (Group D), Group III: ICBM scaffold and rhBMP-2 without bone marrow (Groups E-G) and Group IV: ICBM scaffold only (Group H) (Table 2-5).

2.2.2.2. Preparation of specimens for an implantation

A. PRP preparation

Nine hundred microliters, 900 μl, of PRP was obtained from 30 ml of whole blood. Whole blood was drawn from 4 male 2 month-old Spaque Dawly rats. PRP was prepared and characterized according to the PRP procedures stated in sections

3.4.13.4.1 and 3.4.2 PRP was kept at RT under mild continuous rotation approximately for 1.5 – 2.00 hours before it was used. PRP, PPP and whole blood were activated or clotted by mixing those blood products with 25 unit Bovine thrombin in a 0.5% $CaCl_2$ solution (See platelet activation procedure in section 3.4.3).

B. Bone marrow preparation

Bone marrow was harvested from 8 femurs and 8 tibias from 4 rats. Femur harvesting and bone marrow preparation were performed as stated in the Ostgeogenic cell culture section 3.1.1. A minimum amount of culture medium was used to flush bone marrow. The total volume of bone marrow from one rat was adjusted to 300 µl using a culture medium. The culture medium used in this part of study was DMEM-F12, 10% FBS and 1% Penicillin-Streptomycin and 0.1% Amphotericin B.

C. ICBM scaffold preparation for implantation

(1) Group I: ICBM scaffold with bone marrow and blood products

Five hundred microlitres of mixtures of bone marrow and PRP, PPP or whole blood were prepared for implantation in 5 mice. Three hundred microliters (300 µl) of bone marrow from one rat was mixed with a whole PRP from one rat, 200 µl, or 200 µl of PPP or 200 µl of whole blood. One hundred microliters (100 µl) of each 500 µl mixture was seeded on one ICBM scaffold.

Therefore 60 µl of bone marrow and 40 µl of PRP, PPP or whole blood were seeded on each 5 x10 mm ICBM scaffold. Forty microlitres of PRP contained $2.5x10^8$ platelets. Bone marrow and PRP from one rat were implanted in 5 mice or 20% of bone marrow and 20% of PRP from one rat were implanted in one mouse (Figure 2- 6). For a detailed procedure of fresh bone marrow seeding on an ICBM scaffold see section 3.2.2.2.

(2) Group II: ICBM scaffold with bone marrow and rhBMP-2

In this group, 1 µg of rhBMP-2 and 60 µl of bone marrow were seeded on each ICBM scaffold. Bone marrow and ICBM scaffolds were prepared as they were performed in groups of bone marrow and blood products (See section 3.2.2.2b). The scaffolds were transferred to the animal house for an immediate implantation.

(3) Group III: ICBM scaffold and rhBMP-2 without bone marrow

Ten, three and one micrograms of rhBMP-2 were lyophilised on 5x10 mm ICBM scaffolds, with five scaffolds per group. The scaffolds were lyophilized at -45°C for 12 hours. The lyophilized scaffolds were implanted without an addition of bone marrow (See lyophilization procedure in section 3.7).

(4) Group IV: ICBM scaffold

Lyophilized ICBM scaffolds were implanted without an addition of bone marrow or rhBMP-2 (For detail procedures of ICBM scaffold preparation see section 3.6).

2.2.2.3. Implantation

In order to exclude interfering effects of osteoprogenitor cells and osteoblasts residing in surrounding bone, periosteum and endosteum and provide optimal environment for cells to grow in rich rich blood supply environment, ICBM scaffolds were implanted intramuscularly in the thigh muscle of 2 month-old nude mice for 4 weeks. One scaffold was implanted in one mouse on the left side. Except in the negative control group, two scaffolds were implanted in one mouse, on its right and left sides. There are five samples in each group. A total of 39 scaffolds were implanted in 37 nude mice (Table 2-5) (For detail implantation procedure, see section 3.4.4.3).

An implantation was performed in three days. An implantation of experimental Group III: ICBM scaffold and rhBMP-2 (Groups E-G) and the negative control group (Group H) were performed on the first day. On the second day, implantation of experimental Group I, bone marrow and blood products (Groups A-C), was performed. Implantation of Group II, bone marrow and rhBMP-2 (Group D), was performed on the third day.

2.2.2.4. Explantation and evaluation

At 4 weeks after implantation, mice were sacrificed in a carbon dioxide gas chamber. The implant was carefully dissected from the muscle and immediately

radiographed using Faxitron at 25 Kvp, 35 seconds. Then they were fixed in 10% buffered formalin (For explantation procedures see section 3.4.4.5).

One representative specimen showing an average amount of bone formation in the radiographs from each group was selected for decalcified paraffin embedded histological specimen. The rest of the specimens were selected for non-decalcified histological specimens.

Patterns of bone formation were evaluated from conventional radiographs and histological specimens. The mineralization area was measured by an NIH image public domain. Giemsa staining, Masson-Trichrome-Goldner, Toluidine blue and von Kossa stainings were performed. Histological specimens were viewed under a light microscope (See evaluation procedures of bone formation in section 3.5).

Table 2- 5 Groups of study in PRP *in vivo* implantation

Groups	Characteristics	Numbers of ICBM/mice	Numbers of mice/group	Numbers of ICBM / group	Implantat-ion time
Experimental group I: ICBM scaffold + Bone marrow + blood products					
A	ICBM scaffold + Bone marrow + whole blood	1	5	5	28 days
B	ICBM scaffold + Bone marrow + PRP 100%	1	5	5	28 days
C	ICBM scaffold + Bone marrow + PPP	1	5	5	28 days
Experimental group II: ICBM scaffold + Bone marrow + rhBMP-2					
D	ICBM scaffold + Bone marrow + 1 μg rhBMP-2	1	5	5	28 days
Experimental group III: ICBM scaffold + rhBMP-2					
E	ICBM scaffold + 10 μg rhBMP-2	1	5	5	28 days
F	Scaffold + 3 μg rhBMP-2	1	5	5	28 days
G	Scaffold + 1μg rhBMP-2	1	5	5	28 days
Negative control group					
H	Scaffold only	2	2	4	28 days
Total			**37 mice**	**39 scaffolds**	

Figure 2- 5: Study design of an *in vivo* implantation

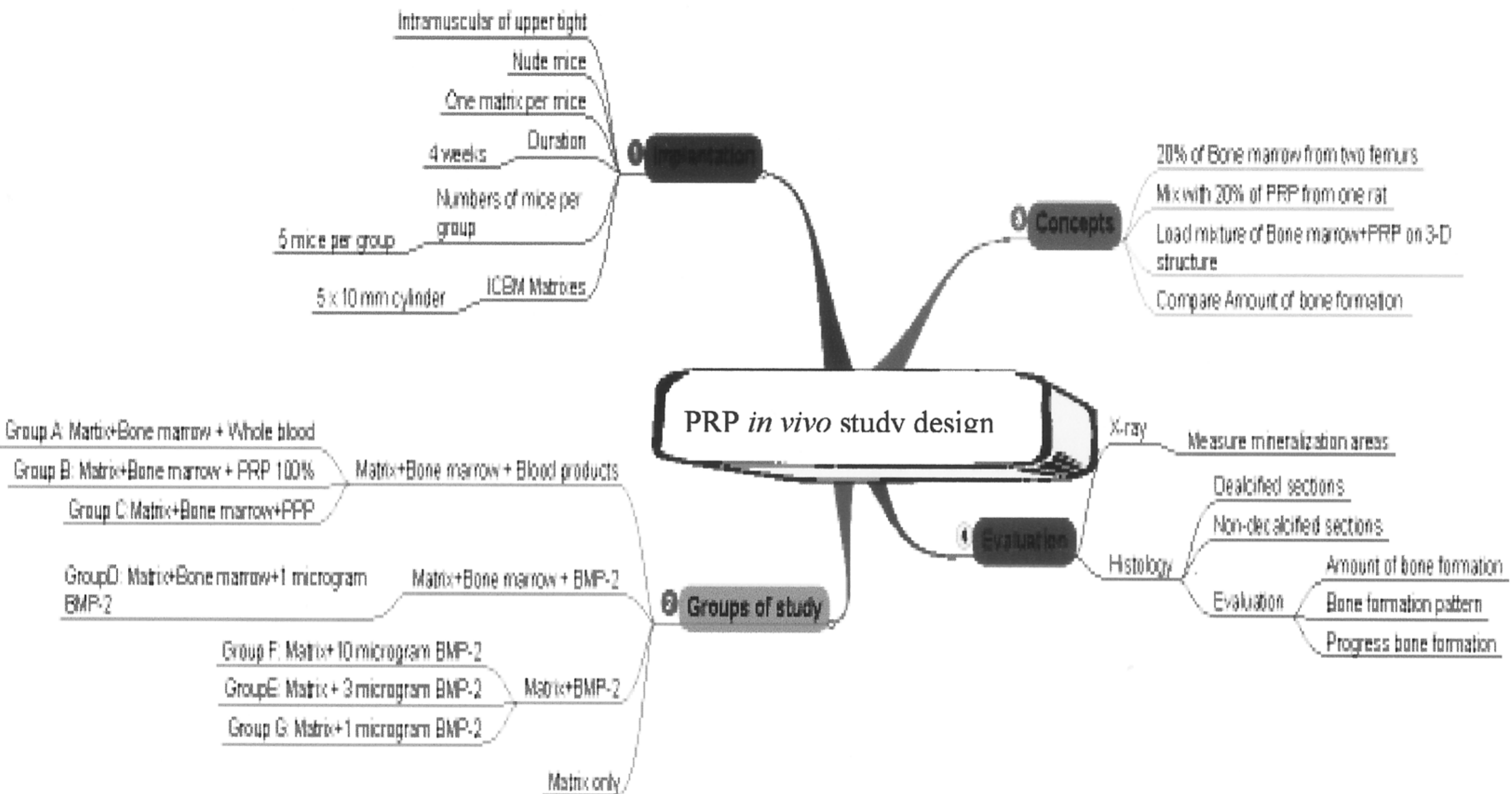
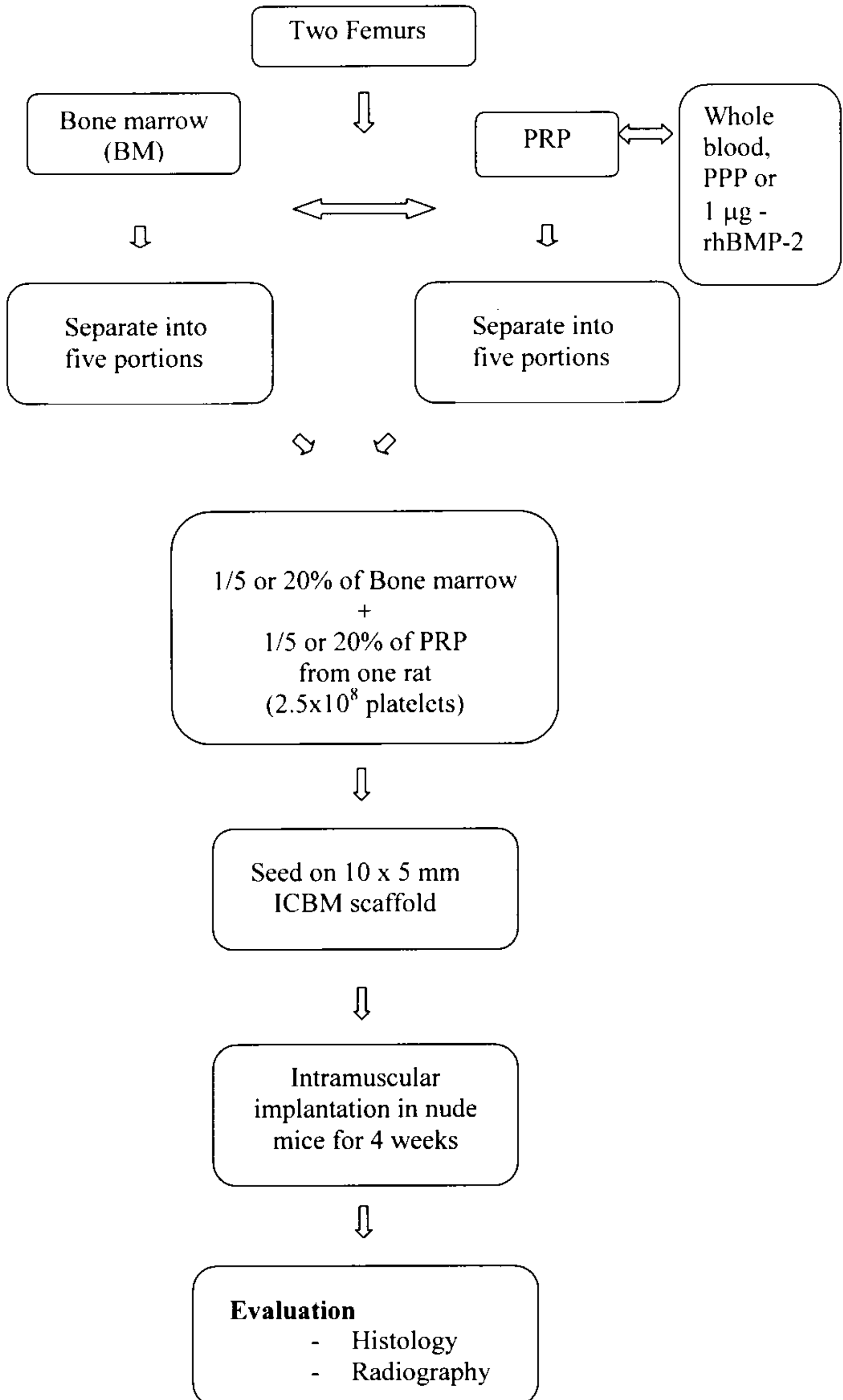


Figure 2- 6 An overview of implantation of bone marrow and PRP *in vivo*



2.2.3. Measurement of TGF-β1 content

Five hundred microliters of PRP, and PPP from *in vitro* and *in vivo* experiments (total of 3 samples) were collected (Table 2- 6). They were mixed with 83.5 μl of 1000 units of Bovine thrombin and 10% $CaCl_2$ solution in 1.5 ml Eppendorf cups. Eppendorf cups were placed on a Eppendorf thermomixer with gentle vibration at 37°C for one hour. Then they were incubated at 4°C for 24 hours. The clots were centrifuged (Joun Bi) at 1000g or 2300 rpm at 4°C for 30 minutes. Supernatant was collected and kept at -70°C for an analysis (Landesberg et al., 2000).

The samples were thawed on ice immediately before analysis. The samples were activated with 2.5N Acetic acid/10M urea and 2.7 N NaOH/1M HEPES. The activated PRP was diluted to 1:600 and the activated PPP was diluted to 1:24 for measurements of activated TGF- β1 content, using the TGF- β1 detection kit, ELISA, R&D system. The procedures were performed as suggested in the manufacturer's guideline of the commercial assay kit. The substrate produced a yellow colour solution. Optical density was determined within 30 min at 450 nm and the correction wavelength was set at 540-570 nm. Concentrations of TGF-β1 in samples were correlated with a curve of standard solutions of TGF-β1.

Table 2- 6 Samples and dilution factors used in TGF-β1 assay

Sample numbers	Samples	Dilution factor
1	500 μl of **PRP** from a set of WST/Morphology study groups *(on the first day of in vitro experiment)*	1: 600
2	500 μl of **PRP** from a set of ALP study groups *(on the second day of in vitro experiment)*	1: 600
3	500 μl of **PRP** from *in vivo* implantation groups	1: 600
4	500 μl of **PPP** from a set of WST/Morphology study groups *(on the first day of in vitro experiment)*	1:24
5	500 μl of **PPP** from a set of ALP study groups *(on the second day of in vitro experiment)*	1:24
6	500 μl of **PPP** from in vivo implantation groups	1:24

3. Procedures

3.1. Primary cell culture of rat bone marrow

3.1.1. Osteogenic cell culture

Immediately after Spraque Dawley rats were sacrified in a carbon dioxide gas chamber, femurs were harvested. Both end of the femurs were intact and were covered with surrounding muscles. They were immersed in Betadine solution (Braunol) in a sterile container and transferred to a culture hood.

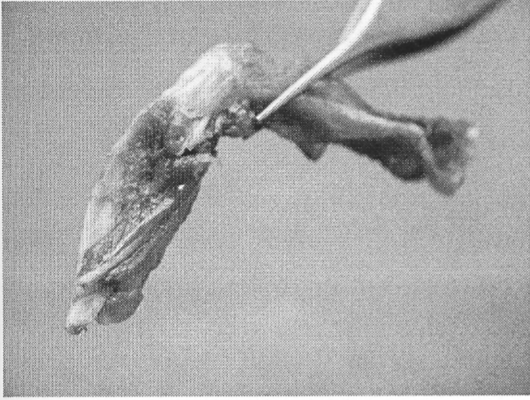
Under the culture hood, on a sterile surface, muscles were removed from femurs. Both ends of the femurs were opened using bone cutting forceps. Small needles, Gauge 20, were moved along the inner wall of the femur to loose marrow from the bony wall. Culture medium, 20-25 ml, loaded in a 5 ml disposable syringe, was injected into femurs to flush bone marrow into a 100 mm culture plate (Figure 2-7).

After that, bone marrow was repeatedly drawn up and down approximately three times using a 5 ml disposable syringe without a needle followed by use of a needle Gauge 19. The solution was kept in a 50 ml Falcon tube, 30 ml in each tube and centrifuged at 1000 rpm for 10 minutes. Supernatant was removed.

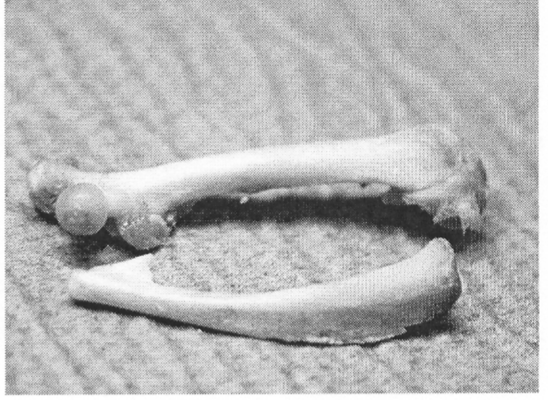
Bone marrow from one rat was suspended in 13 ml of medium. Bone marrow solution was gently pipeted using a 10 ml pipette to create a homogenous solution. The solution was transferred into a 35 mm culture plate or a 100 mm culture plate, 2 ml or 12 ml respectively for incubation. Bone marrow was cultivated in a mineralized medium supplemented with 20 nM dexamethasone and was incubated in 5% CO_2 at 37°C.

The medium was changed at 48 hours after cell seeding and then every 2-3 days. Growth factors were added in the culture medium according to study designs, otherwise, bone marrow was cultured in a standard mineralized medium, DMEMF-12, 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid, supplemented with 10% FBS, 1% penicillin-streptomycin, 0.1% amphotericin B, and 20 nM dexamethasone (Beresford et al., 1994; Cheng et al., 1994; Leboy et al., 1991; Rickard et al., 1994).

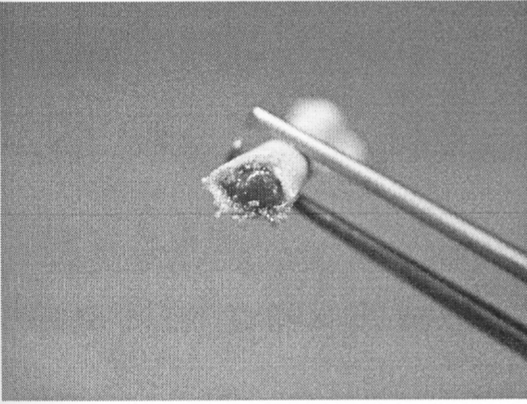
Figure 2- 7 Femur harvesting and bone marrow procurement procedures



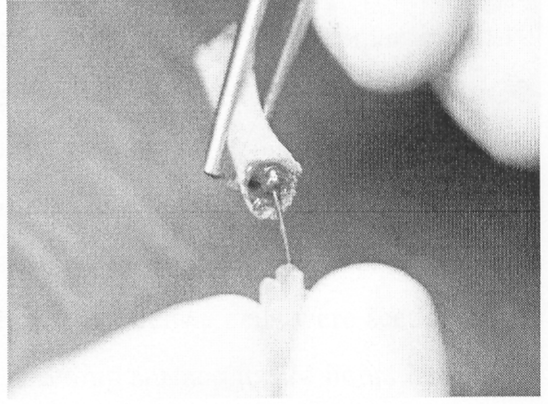
(a) Rat femur covering with muscle



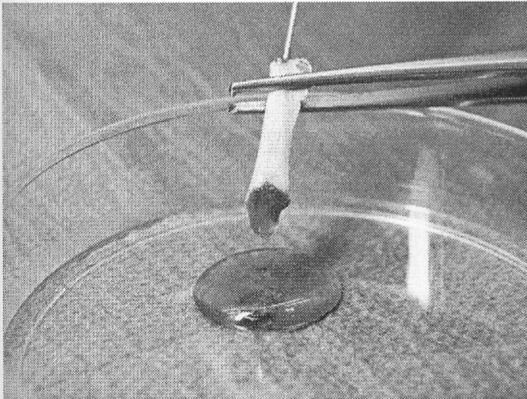
(b) Femur and tibias with closed ends



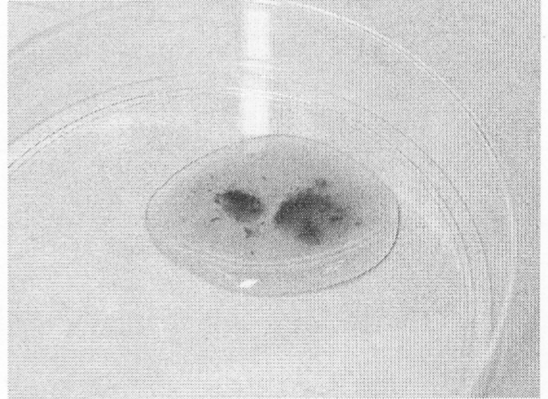
(c) Decapitulation of both ends of femur shaft with bone cutting forcep



(d) Moving a small needle along inner wall of femur to loose its content



(e) Flusing bone marrow out with culture medium



(f) Bone marrow obtained from one femur before performing single cell separation

3.2. Cell seeding

Cell seeding was performed when cells were subcultured or when morphological, histochemical or immunohistochemical stainings and three-dimensional cell culture were to be performed. Fresh bone marrow was seeded on the ICBM scaffold for an implantation *in vivo*.

3.2.1. Two dimensional cell seeding

In the first passages, 5×10^4 cells in 2 ml of medium and 3×10^5 cells in 12 ml of medium were seeded on 35 mm and 100 mm culture plates, respectively.

For staining purposes, steriled 18 x 18 mm glass coverslips were placed on a 35 mm culture plate. A small volume of cell solution, 60 – 70 µl, containing 2×10^4 cells was pipeted on the surface of the coverslips and incubated for 3 hours in 5% CO_2 at 37°C, after that 2 ml of culture medium was added in the 35 mm plate. In chamber slides, 1×10^5 cells in 1.5 ml of culture medium were seeded into each chamber. For an immunohistochemical staining, surfaces of cover slips or chamber slides were treated with Poly-D-Lysin 0.1 mg/ml (50 µl / 1 cm^2) before cells were seeded on the surface. Seeded cells were cultivated on the seeding surface for 24 hours before cell staining (Table 2- 7).

Table 2- 7 List of cells seeding densities on different cell culture containers

Containers	Surface area (cm^2)	Numbers of cells seeding	Cell seeding density
100 mm culture plate	58.0	3.0×10^5 cells/plate	5×10^3 cells / cm^2
35 mm culture plate	9.6	5×10^4 cells / plate	5×10^3 cells / cm^2
Chamber slides	4.2	1×10^5cells/chamber	2.4×10^4 cells / cm^2
18x18 mm coverslips	3.24	1.7×10^4 cells / slip	5.2×10^3 cells / cm^2

3.2.2. Three-dimensional cell seeding

3.2.2.1. Seeding of differentiated cells on 5x3 mm ICBM scaffold for cell culture in PRP study

All bone marrow cells in primary passage at the 6th-8th culture-day were pooled to make cell solution at a concentration of 2×10^6 cells / ml. Twenty microliters of 2×10^6 cells / ml containing 4×10^4 cells were seeded on the upper surface

of moistened neutralized 5x3 mm ICBM scaffolds (Figure 2- 8 a). Cells were allowed to attach on ICBM scaffolds in a small volume of culture medium for 3 hours in 5% CO2 at 37°C. Then 1.5 ml of culture medium was added into each well.

3.2.2.2. Fresh bone marrow seeding for an implantation

A. Bone marrow seeding on 3x5 mm ICBM scaffold (in osteogenic differentiation study)

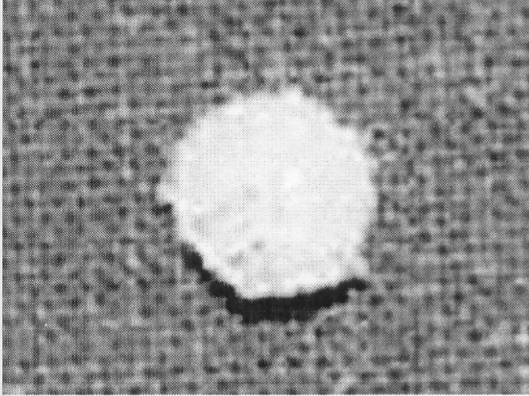
Bone marrow was prepared as described in the osteogenic cell culture section. After centrifugation, the bone marrow pellet, without an addition of culture medium, was separated into 5 portions. One portion was pipeted on one neutralized 3x5 mm ICBM scaffold.

B. Bone marrow seeding on 5 x 10 mm ICBM scaffold (in PRP study)

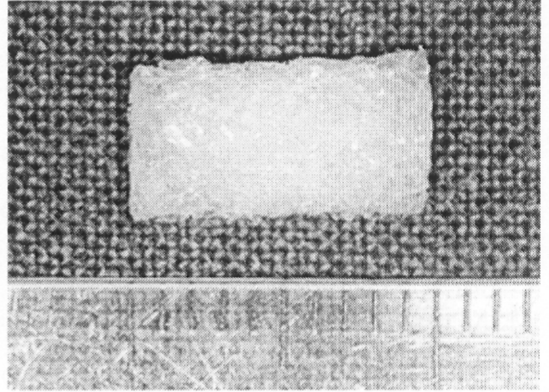
Sixty microliters of bone marrow or 100 μl of mixture of bone marrow and blood products were seeded on a 5x10 mm ICBM scaffold (Figure 2- 8b). Neutralized 5 x 10 mm ICBM scaffolds were loaded into 1 ml syringes, which were cut to a level of 0.5 ml and stood in a 1.5 mm Eppendorf cup, one scaffold in one syringe. Before seeding of bone marrow on scaffolds, 10 μl of 25 unit thrombin in 0.5% calcium chloride solution was pipeted on each scaffold. After that, one hundred microliters of mixture of bone marrow and blood product mixture were seeded on each scaffold (Figure 2- 9). Eppendorf cups were placed in 12 ml polystyrol Falcon tubes and centrifuged at 1000 rpm (492g) (JuanBi) for 2 minutes to distribute the mixtures into a structure of scaffolds. This centrifugation allowed a simultaneous mixing of loaded bone marrow and clotting solution within the structure of scaffolds. Scaffolds were transferred in a tightly closed Falcon tube to the animal house for an immediate implantation (Figure 2- 10).

In Group D: bone marrow and 1 μg rhBMP-2, before an addition of 10 μl of clotting solution, 10 μl of 100 μg/ml rhBMP-2, was pipeted on ICBM scaffolds and allowed to be absorbed for 15 minutes in a closed chamber. After that 10 μl of clotting solution and 60 μl of bone marrow were added on an ICBM scaffold as stated in the previous paragraph.

Figure 2- 8 Inactive insoluble collagenous bovine bone scaffold (ICBM)

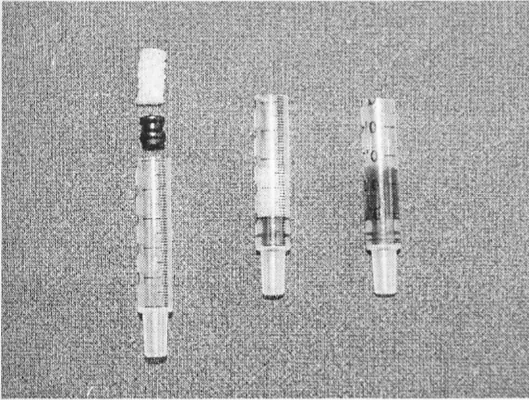


(a) 5x3 mm ICBM scaffold

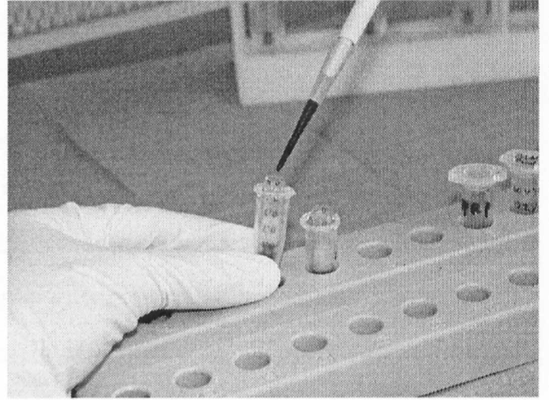


(b) 5x10 mm ICBM scaffold

Figure 2- 9 Bone marrow cell seeding on 5x10 mm ICBM scaffolds for implantaion

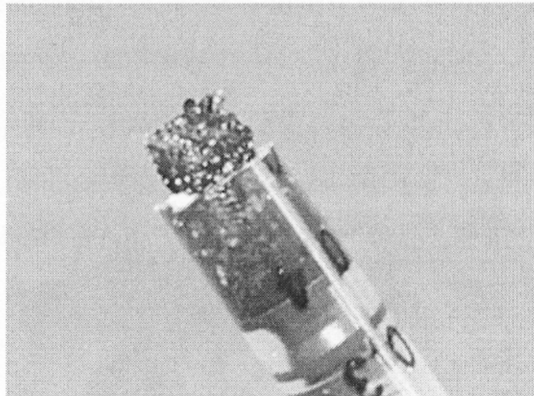


(a) Loading of 5 x 10 mm ICBM scaffold in 1 ml syringe



(b) Pipetting bone marrow into scaffold for centrifugation

Figure 2- 10 Bone marrow and cloted blood within structure of 5x10 mm scaffold



3.3. Characterizing types and growth of differentiated cells

3.3.1. Histochemical staining

3.3.1.1. Alkaline phosphatase staining

Alkaline phosphatase staining was performed on cells seeded on 18 x 18 mm coverslips and 3x5 mm ICBM scaffolds (See section 3.2.1). Fixing solution, Sodium nitrite-FBB a lkaline s olution a nd s taining p rocedures w ere p repared a nd p erformed following the manufacturer's guideline from a alkaline phosphatase staining kit (Sigma). Counter staining with neutral red 0.05% was performed only in two-dimensional cell culture. Coverslip was mounted on a glass slide with glycerine gelatine mounting media at 45°C. Staining was observed under a light microscope (Wada et al., 1998).

3.3.2. Immunohistochemical stainings of collagen type I and osteocalcin

Cells were seeded on 18 x 18 mm coverslips or on chamber slides (See detail of cell seeding in section 3.2.1). Cells were fixed in -20°C acetone for 10 minutes. R.T.U. V ectastain U niversal E litee A BC p eroxidase kit w as u sed a nd t he s tainings were performed following the manufacturer's guideline (Vector Laboratories). An incubation in horse serum to block non-specific staining was skipped for cell culture staining.

A primary antibody was diluted just before use in an antibody diluting solution, 1% Bovine serum albumin and 0.1% NaN_3 in DPBS. Monoclonal antibody, mouse anti-Collagen type I (Sigma), was diluted to 1:20,000 and monoclonal antibody, mouse anti-Osteocalcin (Takara Shuzo), was diluted to 1:50,000. Cells were incubated with the primary antibody at 4°C for 12 hours.

They were counter stained in Weigart hematoxylin for 1 minute and blue in running tap water for 1 minute. Stainings were covered with a cover slip using glycerine gelatine gel at 45°C (Hanada et al., 1997; Wada et al., 1998; Zohar et al., 1998).

3.3.3. Morphological stainings

Cells were incubated on 18 x 18 mm coverslips, 35 mm culture plate or chamber slides or a 3x5 mm ICBM scaffold at least 24 hours after cell seeding (See cell seeding section 3.23.2 for detail), before they were stained. Cells were washed in DPBS and fixed in 10% buffered formalin for 5 minutes or otherwise as stated in each protocol. Coverslips were mounted using glycerine gelatine gel at 45°C. Cell morphology and staining were observed under a light microscope.

3.3.3.1. Alcian blue staining

This staining was to detect mucopolysaccharide in cytoplasm of chondroblast and extracellular matrix of mesenchymal cells. Cells were covered with 3% acetic acid for 3 minutes. Acetic acid was removed and Alcian blue solution was added on the surface of cells for 30 minutes at RT. Cell surface was rinsed in distilled water. Cells were counter stained in a nuclear fast red for 5 minutes and washed in tap water. Light blue staining in an extracellular scaffold and/or cytoplasm was interpreted as positive staining. Red staining of cytoplasm of cells was negative staining (Beresford et al., 1994; Cheng et al., 1994).

3.3.3.2. Neutral red vital staining

The staining is to demonstrate vital cells which attach on surfaces of scaffolds or cell culture plates. Neutral red stains the nucleus of vital cells. Cells were incubated in 0.05% Neutral red solution in 5% CO_2 at 37°C for 2 hours. The solution was removed and a fresh medium was filled in the culture plates or wells. The nucleuses of vital cells stained red (Lindl and Bauer, 1989).

3.3.3.3. Oil red O staining

This staining was performed to detect fat cells by staining lipid droplets in the cytoplasm of cells. Cells were incubated in 1 ml of propylene glycol two times, for 5 minutes each. Two milliters of Oil red O was added with continuous agitation for 7 minutes using a shaker (Desaga). After that, they were incubated in 85% Propylene glycol for 3 minutes then they were rinsed in distilled water. Cells were counter stained in Weigart hematoxylin for 1 minute then they were blue in running tap water.

Bright red staining of lipid droplets in cytoplasma was observed as positive staining under a light microscope (Beresford et al., 1994; Cheng et al., 1994).

3.3.3.4. Co-staining of ALP and Oil red O staining

Immediately after staining with sodium nitrite and FBB-Alkaline solutions (Sigma), cells were rinsed with deionized water. Oil red O staining was performed as stated in the Oil red O staining section. Co-expression of cells with blue cytoplasmic staining and red lipid droplets in cytoplasm were observed under a light microscope (Beresford et al., 1994; Cassiede et al., 1996).

3.3.3.5. von Kossa staining

The stain was used to detect mineralization of extracellular matrix (ECM). Cells were covered with a 5% silver nitrate solution then they were placed under ultraviolet light (Exakt-light-polymerization unit) for 1 hour. Cells were washed in tap water, for 3 times, then, they were incubated in sodiumthiosulfate solution for 5 min. The staining was washed in tap water for three times and incubated in 2 ml of nuclear fast red for 5 minutes to counter stain. After that, they were thoroughly washed in running tap water. A black stain on mineralized ECM was interpreted as positive staining and a bright pink stain of cytoplasm as negative staining (Harris et al., 1994; Wada et al., 1998).

3.3.4. Bioassays

3.3.4.1. Vitality of cell detected by WST assay (Roche)

WST assay measures cell proliferation and cell viability. The assay determines the number of viable cells by cleavage of tetrazolium salts added to culture medium. The tetrazolium salts are cleaved to formazen by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in an amount of formazan dye formed which directly correlates to the number of metabolically active cells in the culture. The formazen dye is quantified by spectrophotometry.

Differentiated bone marrow cells, $4x10^4$ cells, were seeded on 3x5 mm ICBM scaffolds and placed in a 24 well plate. Cells were washed in DPBS and incubated in 550 μl of 10% WST in the culture medium in 5% CO_2 at 37°C for one hour. An incubation of 10% WST without cell in 5% CO_2 at 37C for 1 hour was used as a background control of the measurement.

Two hundred microliters of supernatant were pipeted into each well of a 96 well culture plate. The measurement was performed in doublet, one sample in two wells. The optical density was measured at 450 nm (TECAN rainbow Spektralphotometer-Spectronic 301, Germany). The optical density of background control was deducted from the read optical values of samples. Average values of double measurements were used (Merklein et al., 1998).

3.3.4.2. ALP activity measurement

The assay measures ability of the enzyme to convert p-nitrophenylphosphate to p-nitrophenol. The activity of the enzyme was measured as μM of nitrophenol (Wada et al., 1998).

A. Cell lysis

Cells on ICBM scaffolds were washed with DPBS and kept at -20°C for analysis. Cells were subjected to repeated freezing and thawing three times for 30 minutes at each cycle. Then 500 μl of 2% Triton X-100 in PBS at 4°C was added into each well of the 24 well-plate on ice. The ICBM scaffolds, cells on the floor of the culture plate and 2% Triton X-100 in PBS were transferred to microcentrifuge tubes on ice. They were vigorously vortexed for 1 minute. The solution was incubated on ice for 90 minutes then they were again vigorously vortexed for 1 minute and centrifuged at 1000 rpm (Jouan BR4i) at 4°C for 15 minutes to remove any bubbles in the solution. Supernatant was pipetted into a new 1.5 ml microcentrifuge tube for further ALP activity measurement (Rozycki and Edelstein, 1996).

B. Measuring of ALP acitivity

Just before use, 2 mg of p-Nitrophenylphosphate was added in 1 ml of lysis solution (0.75 mM 2-Amino-2-methyl-1-propanol, pH 10.3) and mixed well. The solution was used as a lysis buffer. One hundred microliters of protein solution from

Triton X-100 cell lysis and 100 μl DPBS were pipeted into a 1.5 ml Eppendorf cup, then 400 μl of lysis buffer was added into each cup. They were incubated at 37°C in an Eppendorf thermomixer with gentle vibrating for 1 hour. After that, the reaction was stopped by adding 400 μl of 50 mM NaOH into each cup and the solutions were further diluted by adding 100 μl of deionized water. They were vortexed shortly and centrifuged at 1300 rpm (Biofuge 13) for 5 minutes to obtain a clear supernatant.

Various intensities of yellow solution were interpreted as a positive reaction. Two hundred microliters of supernatant were added in each well of the 96 well-culture plate. Light absorbance was measured at 405 nm using the TECAN rainbow. A concentration of ALP in each sample was interpreted from standard curve of p-nitrophenol, 5-250 μM, standard solutions. The measurements of each sample were performed in triplet, one sample in three wells (Wada et al., 1998).

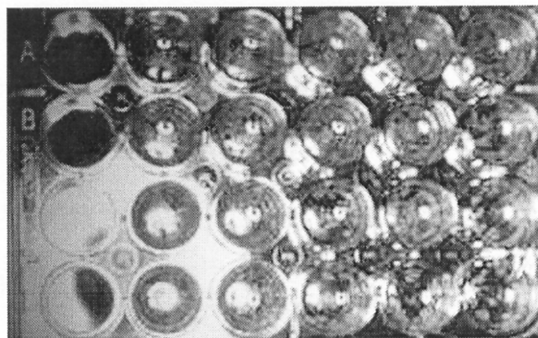
3.3.4.3. Measuring of calcium content in extracellular matrix

An assay aims to measure the amount of calcium content in a mineralized extracellular matrix of cell culture. The principle is that calcium in test solution will combine with o-Cresolpthalein complexone in an alkaline medium. The reaction yields Calcium-Cresolphthalein complexone complex, which has a red colour. An intensity of red colour will be measured and correlated with a concentration of calcium in standard mediums. In this study, the calcium content was measured only in three-dimensional cell culture using the Calcium Kit (Sigma diagnostic).

A. Calcium solubilization

ICBM scaffolds, which were used in the WST assay, were washed with Tris pH 7.5, before they were use in analysis of calcium content. The scaffolds were minced into small pieces, 1 mm, using a sharp surgical blade and placed in a 24 well plate. Five hundred microlitres of 0.5 M HCL was added into each well. They were incubated under continuous vibration for 12 hours at RT (Figure 2- 11). Then the supernatant was pipeted to 1.5 ml Eppendorf cups and was centrifuged at 1300 rpm (Biofuge 13) for 5 minutes to obtain clear supernatant. The supernatant was pipeted into new Eppendorf cups and kept at 4°C for further analysis.

Figure 2- 11 Solubilization of calcium content in mineralized extracellular scaffold



(a) An incubation of small pieces of scaffolds in 0.5 M HCl for 24 hours at RT

B. Measurement of calcium content

The calcium kit and Calcium/Phosphate standard set (Sigma diagnostic) were used. The measurement was performed following the manufracturer`s guideline. Six concentrations of calcium contents of standard solutions, 25, 50, 100, 125 and 150 mg/l were prepared. Hydrochloric acid, 0.5 M, was used as negative control. Ten microliters of standard solution, negative control, and samples were added into each 1 ml of working solution.

Two hundred microliters of working solutions were added into a 96-well plate. The optical densities were measured at 570 nm using the TECAN rainbow. Optical densities of samples were correlated with an optical density curve of standard solutions for an interpretation of the calcium contents. The measurements of each sample were performed in triplet (Hanada et al., 1997; Hay et al., 1999).

3.3.5. Procedures in molecular biology

3.3.5.1. Messenger RNA (mRNA) extraction

MicroPoly(A) Pure Kit (Ambion) was used to isolate poly(A+)mRNA directly from eukaryotic tissue or cells (10-5x10^6 cells or 100-200 mg or total RNA of 2-400 µg). The components of MicroPoly(A) Pure Kit were demonstrated in Table 2- 8.

In this study mRNA was extracted from a pellet of $1x10^5$ cells. Fifteen microlitres of EDTA H_2O was added into each mRNA pellet. mRNA solution was stored at -70°C.

Table 2- 8 Components of mRNA extraction kit (MicroPoly(A) Pure (Ambion))

Reagents	Number / Amount per package
100 mg Permeasured Oligo dT	20 tubes
Microfuge tubes	20 tubes
Microfuge tubes with spin columns	20 tubes
Lysis solution	60 ml
Binding buffer, for high salt washes and for use with total RNA sample	125 ml
Wash buffer, for low salt washes	125 ml
Dilution buffer (10mM Tris pH 7.5, 1mM EDTA)	120 ml
Elution buffer (10MM Tris PH7.5, 1 mM EDTA)	5 ml
Nuclear-free Water EDTA (DEPC H_2O, 0.1 mM EDTA)	1.0 ml
Glycogen	100 µl

3.3.5.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

The Titan one tube RT-PCR system (Roche) was used. Master mix I and Master mix II were prepared according to the manufacturer's guideline as demonstrated in Table 2-9. Sequences of bases in upstream and down stream primers were demonstrated in Table 2-10. The components were thawed on ice.

One microliter of mRNA, 1 µl each of 20 µM upstream and down stream primers, 23 µl of Master mix I and 25 µl of Master mix II for 1 reaction, were added into 0.2 ml thinwalled PCR tube on ice and vortex, briefly for mixing. Thinwalled PCR tubes were placed in a PCR thermocycle. mRNA and primer templates were annealed at 50°C, denaturated at 94°C and elongated at 70°C for 10 and 25 cycles.

Table 2- 9 Components of Titan one tube RT-PCR system (Roche)

Component	Volume (µl)	Final concentration in the RT-PCR
Master mix I:		
dNTP 10 mM (mixture of 100 mM dATP, dCTP, dGTP, and dTTP)	1	0.2 mM
Downstream primer 20 µM	1	0.4 µM
Upstream primer 20 µM	1	0.4 µM
Template RNA	1	1 µ´g – 1 pg total RNA
DTT solution (100 mM)	2.5	5 mM
RNase inhibitor (40 U/µl)	0.5	5 – 10 Unit
Distilled water	18	
Total volume in one tube	25	
Mastermix II:		
5 x RT-PCR buffer with Mg^{2+}	10	1.5 mM $MgCl_2$
Enzyme mix	1	AMV and Expand high fidelity PCR-System
Distilled water	14	
Total volume in one tube	25	

Table 2- 10 Sequences of bases of upstream and downstream primers

Primers	Sequences	Expected base pairs	Gene bank access number
ALP			
Upstream	5′- CGG ACC CTG CCT TAC CAA CTC ATT TGT GCC – 3′	336	J03572.1
Downstream	5′- CGC ACG CGA TGC AAC ACC ACT CAG G – 3′		
BMP-2			
Upstream	5′- CCA CGA CGG TAA AGG ACA TCC ACT CC – 3′	338	NM-017178
Downstream	5′- GTG GAG GGT TGC GGG TGT CGC TAG – 3′		
Collagen type I			
Upstream	5′- CTC CCT GGC CCC CCT GGT GCA CC – 3′	348	Z78279
Downstream	5′- CCC TGG ACC CCC TGG CAC TGC TGG TGC TC – 3′		
GAPDH			
Upstream	5′- CCA CGA GAA ATA TGA CAA CTC CCT C -3′	348	AF106860
Downstream	5′- GGT GGT GAA GCA GGC GGC CGA GGG – 3′		
Osteocalcin			
Upstream	5′- GAG GAC CCT CTC TCT GCT CAC TCT GCT GG – 3′	341	X04141
Downstream	5′- CCT CTC TCT GCC TCG AAA GTA TGG AC – 3′		

3.3.5.3. Gel electrophoresis

Gel electrophoresis was performed to detect c-DNA in RT-PCR products. A gel running tray, Model B2 (Owl Separation systems. Inc) with 5x2 mm combs was used. PCR products were run on a 0.2% Agarose gel under constant 100 volts for 1 hour. Superladder-mid1 100bp (Roth) was used as a base pair marker.

The cDNA in the gel was observed by immersing the gel in 1% Ethydium bromide for 10 minutes and visualized under ultraviolet light (Biorad-electrophoresis light machine). Sharp dark bands at a level around 400 bp were read as positive bands. The images were captured and stored by Gel analysis softwear, BioRad-Multi-Analyst.

Intensities of positive bands of samples were measured by using the public domain NIH image software on a personal computer. The intensities were normalised by that of the house keeping gene, GDPH, on the same gel. The results were reported as density ratios of samples to that of the GDPH (Fromigue et al., 1998). Methods used to characterize types and growth of differentiated cells are summarized in Table 2- 11.

Table 2- 11 Methods to characterize types and growth of differentiated cells

Parameters	Variables	Methods	References
Cell growth	Cellular metabolic activity	WST assay	(Merklein et al., 1998)
Osteoblastic differentiation	Alkaline phosphatase (ALP) activity	Measurement of ALP activity using bioassay	(Wada et al., 1998)
		ALP histochemical staining, using ALP staining kit (Sigma)	(Wada et al., 1998)
		Expression of ALP mRNA, using RT-PCR (Roche)	(Fromigue et al., 1998)
	BMP-2	Expression of BMP-2 mRNA, using RT-PCR (Roche)	(Fromigue et al., 1998)
	Type I collagen	Type I collagen in ECM, using immunohistochemical staining	(Hanada et al., 1997; Wada et al., 1998)
		Expression of type I collagen mRNA, using RT-PCR (Roche)	(Fromigue et al., 1998)
	Osteocalcin	Osteocalcin in ECM, using Immunohistochemical staining	(Hanada et al., 1997)
		Expression of osteocalcin mRNA, using RT-PCR (Roche)	(Fromigue et al., 1998)
	In vitro mineralization	Mineralization of ECM, using von Kossa staining	(Harris et al., 1994; Wada et al., 1998)
		Measurement of calcium content in ECM, using Calcium detection kit (Sigma diagnostics)	(Hanada et al., 1997; Hay et al., 1999)
Adipocytic differentiation	Fat droplet in cytoplasm	Oil Red O staining	(Cheng et al., 1994)
Chondroblastic differentiation	Production of mucopolysaccharide in cytoplasm	Alcian blue pH 2.5 staining	(Cheng et al., 1994)
Co-differentiation of osteoblasts and adipocytes	Expreesion of ALP on cell membrane and fat droplets in cytoplasm	Double staining of ALP and Oil red O stainings	(Cassiede et al., 1996)

3.4. Procedures related to Platelet-rich plasma (PRP)

3.4.1. PRP preparation

3.4.1.1. Blood drawing

Male SD rats, 300 g, were anesthesized by intramuscularly injecting Rompun, 5 mg/kg, and Ketavet, 120 mg/kg. Heart puncture was performed when rats were fully anesthesized. Sodium Citrate-syringe, S-Monovette 5 ml, and Movette Needle, non-pyrogenic, No. 2, were used. The skin of the rat was cleaned with 70% alcohol before heart puncture was performed.

When fully anesthesized the rats were placed in dorsal recumbency. The needle was inserted under the xyphoid cartilage slightly to the left of the midline. The needle was advanced at a 20 to 30 degree angle from the horizontal axis to the sternum to enter the heart. One should aspirate lightly while advancing. Blood was drawn slowly (Animal care unit, 1996).

3.4.1.2. PRP separation procedures

Under the culture hood, all blood was pooled and separated into a 15 ml Polystyrol Falcon tube, 10 ml/tube. The tubes were centrifuged (Jouan BRi) at 1125g (2500 rpm) RT for 10 minutes, then plasma, white blood cell (WBC) and RBCs on the upper surface were separated from the packed RBCs in the bottom level. The plasma was centrifuged (Jouan BRi) at 300g (1300 rpm) in RT for 25 minutes. Concentrate platelets, WBCs and upper part of RBCs located in the middle layer of the centrifuge were separated as platelet-rich plasma (PRP) (Figure 2-12). Plasma located on the upper part of the tube was separated as platelet poor plasma (PPP). PPP was centrifuged at 1000 rpm for 10 minutes. Supernatant was used as PPP in the study (Landesberg et al., 2000).

3.4.2. PRP characterization

3.4.2.1. Platelet counting

The numbers of RBCs, WBCs and platelets in whole blood, PRP and PPP were counted using the blood counter, Casy 1, Schäfe system. PRP was diluted with

isotone into 1:10 of PRP concentration for counting. Whole blood and PRP were also smeared on a glass slide and were stained with May-Gruenwald and Giemsa stainings.

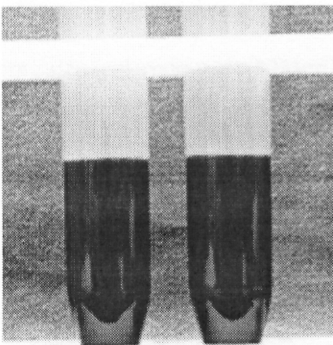
3.4.2.2. Platelet smear

The smears were fixed with light heat. May-Gruenwald was dropped on the glass slide to cover the surface of the smears for 3 minutes at RT. It was rinsed with deionised water then the surface was covered with 10% Giemsa for 10 minunites at RT. The smears were rinsed with deionised water and air dried. A bright blue colour of small granules or platelets, a light browny red of larger round shape RBCs and various types of WBCs were observed under a light microscope (Landesberg et al., 2000; Marx et al., 1998).

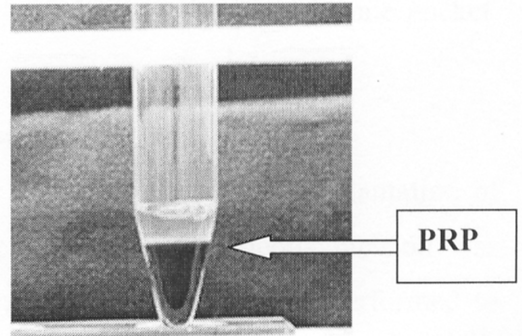
3.4.3. Platelet activation

Platelets were activated by blood clotting. One milliter of PRP, PPP or whole blood was activated with 167 μl of 1000 unit Bovine Thrombin in 10% calcium chloride solution. The two contents were mixed and PRP or other blood products were clotted within one minute after mixing (Marx et al., 1998).

Figure 2- 12 Centrifugation of blood and plasma for PRP preparation



(a) 1st Centrifugation observing separation between plasma and RBC



(b) 2nd Centrifugation observing PRP and WBC in middle layer between PPP on the upper part and RBC in the lowest part

3.4.4. Implantation of ICBM scaffolds in nude mice, subcutaneous and intramuscular implantations

3.4.4.1. Pre-operative care

Nude mice were acclamized for 7 days before any surgical procedure were begun. They were refrained from eating and drinking for 18-24 hours before they were anesthesized.

3.4.4.2. Anesthesia

Mice were anesthesized by intra-peritoneal injection of Rompun, 4 mg/kg, and Ketavet, 100 mg/kg.

3.4.4.3. Implantation

A. Subcutaneous implantation

This procedure was performed in nude mice for an implantation of a 5x3 mm ICBM on the dorsal side of the mice. The skin on the dorsal side from the middle of the back to the tail of the mice was cleaned with 70% alcohol. A horizontal incision of 1 cm long was performed on the right and left side of the posterior one-third of dorsal of the rat, then a subcutaneous pocket, 1 cm deep, was created by blunt dissection. The scaffold was inserted into each pocket, one scaffold in one pocket. The skin was sutured using 4-0 Black silk.

B. Intramuscular implantation

This procedure was performed in male athymic mice for an implantation of 5x10 mm ICBM scaffolds in the thigh muscle. A 1 cm cutaneous incision was performed on the upper part of the left leg. A blunt dissection was performed to expose the muscle bundle. A linear incision parallel to muscle fibres was performed and a blunt dissection was performed to create an intra-muscular pocket. One millilitre of sterile water was injected into the pocket to expand the pocket. Then the scaffold was inserted into the pocket. The muscle layer was closed with 4-0 Vicryl and the skin was closed with 4-0 Black silk.

Post-operatively, mice were wrapped with soft paper and placed under fluorescent light to keep them under a warm temperature in the observing room for a

night. They were transferred to their regular cage the next day. Food and water were supplied immediately after surgery (Figure 2-13).

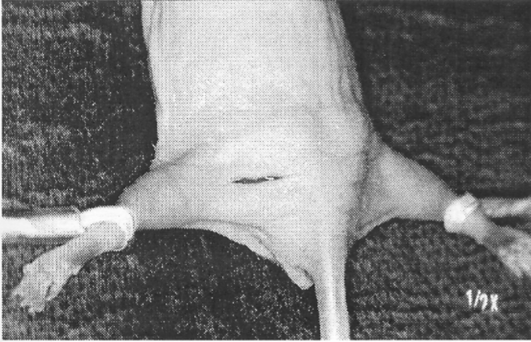
3.4.4.4. Sacrification

Rats or mice were sacrificed in a carbon dioxide gas chamber for 5 minutes. Chest movement was observed as sign of breathing.

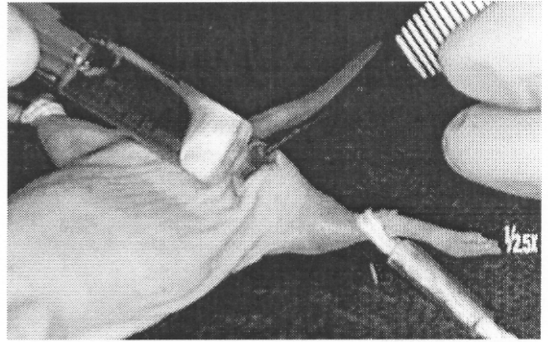
3.4.4.5. Explantation

Skin and m uscle were open using a surgical blade. A blunt dissection was performed to expose implanted specimen. The specimen was carefully removed from the surrounding muscle avoiding damage to the structure of specimen (Figure 2-14).

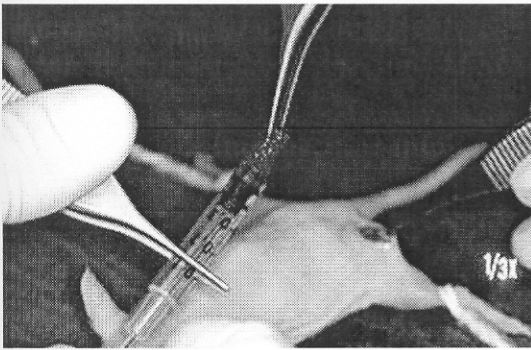
Figure 2- 13 Intramuscular implantation in nude mice



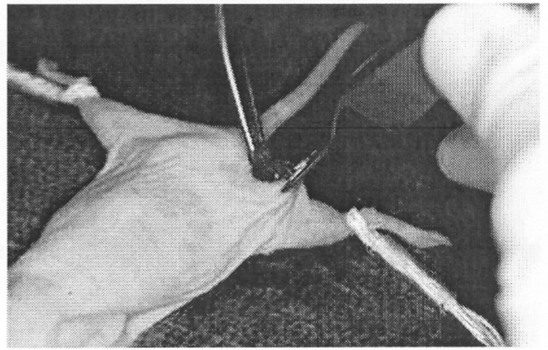
(a) Cutaneous horizontal incision



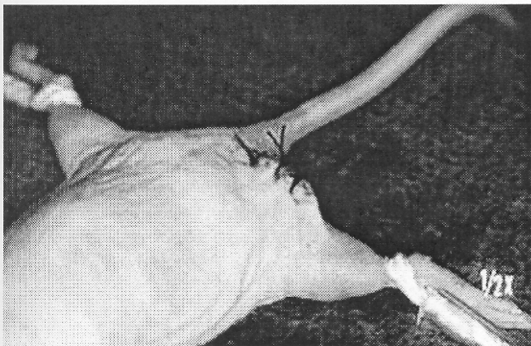
(b) Injecting of sterile water to create intramuscular pocket for an implant



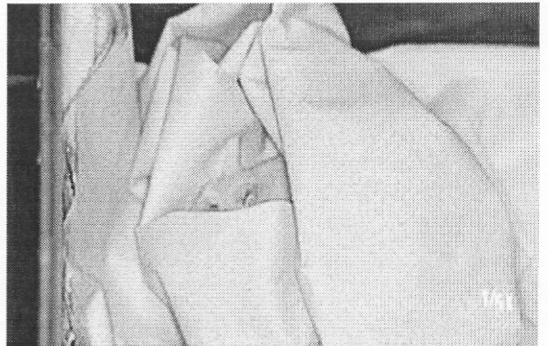
(c) Transfering implant from its container



(d) Inserting implant into created pocket intramuscularly

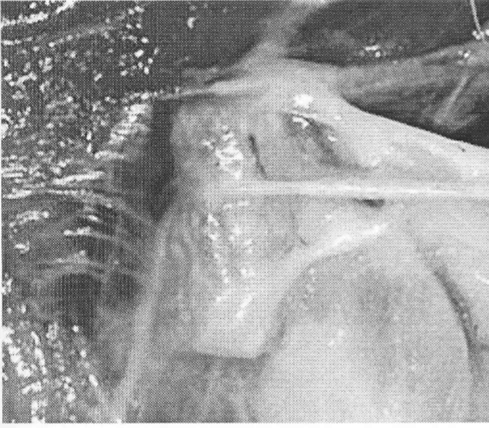


(e) Skin closure using 3-0 Black silk



(f) Keeping mouse in warm temperature during 24 hours after implantation

Figure 2- 14 Explantation of intramuscular implanted specimen



(a) Intramuscular implantation

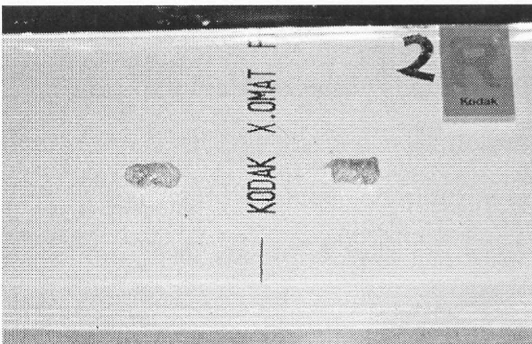
(b) Explanted specimens

3.5. Evaluation of new bone formation

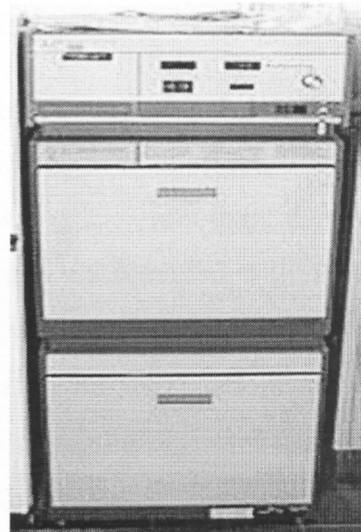
3.5.1. Radiography

Fresh specimens with a minimum amount of surrounding soft tissue were placed on Kodak X-Omat flims. They were radiographed at 25 kV and 35 second using Faxitron (Figure 2- 15).

Figure 2- 15 Specimens prepared for radiographing in `Faxitron´ radiographic machine



(a) Specimens on Kodak X-Omat flim



(b) Radiograph machine `Faxitron´

3.5.2. Histology

3.5.2.1. Histological specimen preparation

Specimens were fixed in 3.5% buffered formalin for at least 24 hours before they were embedded. Decalcified specimens were paraffin embedded and cut into 15 μm thickness and attached on a histological glass slide. Undecalcified specimens were embedded in Technovit 7200 VLC (Donath and Breuner, 1982). The specimens were cut and grinded to 10 μm for histological staining. The Exakt-cutting-grinding system was used to prepare these specimens. Detailed procedures followed the manufacturer's guidelines (EXAKT).

3.5.2.2. Histological staining for non-decalcified specimens

A. Giemsa staining

Filtered Giemsa was diluted with deionized water, at 2:1. Specimen was immersed into the solution for 30 – 45 minutes. The slide was rinsed in deionized water. The intensity of staining was observed under a light microscope. Then the slide was dipped briefly into glacial acid water (8 drops 100% acetic acid in 100 ml distiled water) and washed in running deionized water. The specimen was air dried and coversliped with Technovit 7200 VLC (Donath and Breuner, 1982).

B. Double staining of von Kossa and Giemsa stainings

Specimen were covered with 5% silver nitrate solution and exposed directly under ultraviolet light. The intensity of black staining was observed under a light microscope every three minutes. When the stain was sufficiently dark, the specimen was rinsed with deionized water. Then the specimen was covered with 5% sodium thiosulfate for fixation and washed with deionized water. They were counter stained with Giemsa staining. Giemsa staining was performed as stated in the Giemsa staining section. The specimen was air dried and coversliped with Technovit 7200 VLC.

3.5.2.3. Masson-Trichrom-Goldner staining

The staining differentiated types of cells by stainings of acidophilic, basophilic and neutrophilic structures. Cytoplasm and osteoid stains red, nucleus stains blue or black, bone and collagen stains green, and red blood cells stains orange.

Undecalcified specimen embedded in Technovit 7200 VLC, 10 µm thickness, were immersed in Weigert Eisenhaematoxylin for 20 minutes, then washed in running tap water for 5 minutes (Step I). Step II: the specimen was immersed in Goldner I solution for 7 minutes then washed briefly in 2% Acetic acid. Step III: the specimen was immersed in Orange G for 5 minutes and then washed briefly in 2% acetic acid. Step IV: the specimen was immersed in Light green staining solution for 15 minutes and then washed briefly in 2% acetic acid. Step V: the specimen was washed in deionized water, air dried and coversliped with Technovit 7200 VLC under UV light (Ekxact) for 5 minutes (Donath, 1995).

3.5.2.4. Toluidine blue

A thin section of undecalcified tissue allowed morphological differentiation even in the absence of color differences. The way dye molecules stack up on biological structure can make the stain colour shift to a more purple red. Mineralized bone stains light purple, osteoid stains colourless to pale blue, mineralization front stains light blue, nuclei stains black and mast cells and mucopolysaccharide stain violet.

Undecalcified specimen embedded in Technovit 7200 VLC, 10 µm thick, were immersed in3% acetic acid for 3 minutes and washed in running tap water for 2 minutes. Excess water was dried on absorbance paper. The specimen was immersed in 10% H_2O_2 for 10 minutes and washed in running tap water for 2 minutes. Then the specimen was consecutively immersed in Toluidine blue solution for 5 minutes and washed in running tap water for 2 minutes. The specimen was briefly immersed in 70% ethanol for 20 seconds. The specimen was washed in deionized water, air dried and coversliped with Technovit 7200 VLC under UV light (Ekxact) for 5 minutes. (Donath and Breuner, 1982). Methods used to evaluate new bone formations are summarized in Table 2-12.

Table 2- 12 A summary of methods to evaluate new bone formation

Parameters	Variables	Methods	References
Amount and pattern of bone formation	Mineralization area on radiographs	Measure mineralized area using NIH image software public domain	(Terheyden et al., 1999)
	Histology of bone formation	Decalcified and non-decalcified histological specimens using Giemsa, Masson-Trichrome, Toluidine blue, and von Kossa stainings	(Ohgushi et al., 1993; Terheyden et al., 1999)

3.6. Insoluble collagenous bone matrix (ICBM) preparation

ICBM was produced in the Bone Lab, The University of Wuerzburg, Wuerzburg, Germany under the patent of Kuberasampath and Ridge, US Pat. Nr. 5171574. ICBM was made from bovine bone. Bovine spongiosa was defatted in choloroform and methanol, a 3:1 solution and demineralized in 0.5 M HCL. Extracellular matrix proteins were extracted by 4M GuHCL/50mM Tris-HCL, pH 7.4, at 4°C. The scaffolds were sterilized in chloroform and methanol, a 1:1 solution (Figure 2- 16).

3.7. Lyophilization

3.7.1. Lyophilization of ICBM scaffold

Scaffolds were washed in sterile deionized water. Most of the excess water was sterilily suctioned. ICBM scaffolds were placed on sterile absorbance paper to remove excess water. The scaffolds were placed in 50 ml or 15 ml Falcon tubes and frozen at -70°C for 12 hours, then they were placed in lyophilizator at -45°C for 12 hours or until the scaffolds became completely dry. The scaffolds were kept in tightly closed sterile containers until used.

3.7.2. Lyophilization of growth factors on ICBM

If the growth factors were lyophilised on scaffolds, the lyophilized scaffolds were washed two times in sterile water. Excess fluid was removed. Moistened

scaffolds were placed in 96 well- culture plates, one well for one scaffold, then a growth factor was added on each scaffold. The list of concentration rhBMP-2 solution lyophilized on ICBM is demonstrated in Table 2- 13. The scaffolds were frozen at -70°C for at least 3 hours to freeze fluid on the structure of the scaffold, then they were dried in Lyophilizator at -45°C for 12 hours or until the scaffolds become completely dry. The scaffolds were kept in a closed container at 4°C until they were used (within 14 days).

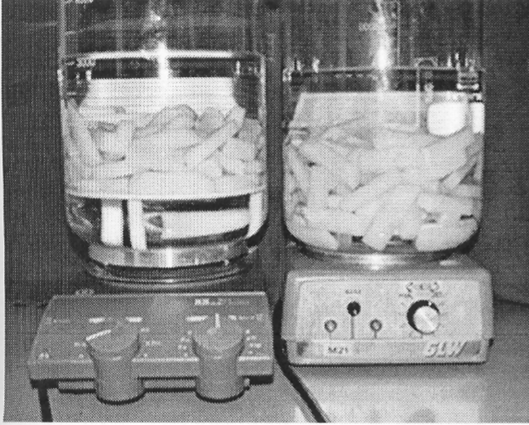
Table 2- 13 List of concentration of rhBMP-2 solution lyophilized on ICBM

Amount of rhBMP-2 on ICBM	Volume of the solution added on ICBM	Concentration of rhBMP-2	Size of ICBM / Usage
300 ng	10 µl	30 µg/ml	3x5 mm / cell culture
1 µg	20 µl	50 µg/ml	5x10 mm / implantation
3 µg	20 µl	150 µg/ml	5x10 mm / implantation
10 µg	20 µl	500µg/ml	5x10 mm / implantation

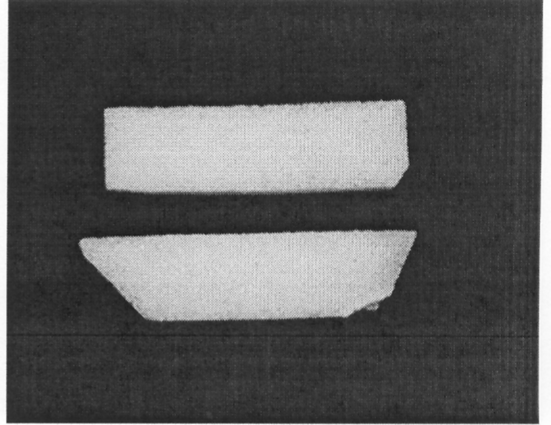
3.8. Neutralization of ICBM scaffold

This step was required when the scaffolds would be used for cell seeding. Lyophilized ICBM without a growth factor was placed in a 50 ml Falcon tube to a level of 20 ml. Culture medium, D-MEM F-12 with 1% Penicillin and Streptomycin and 0.5% Amphotericin B was added to a level of 40 ml. The tubes were centrifuged at 1000 rpm for 10 minutes, for two times. A fresh medium was changed every time after centrifugation. The scaffolds were incubated in fresh medium for 12 hours before they were used. Just before cell seeding, excess fluid was suctioned out and absorbed on sterile filter paper. Scaffolds were placed in a cell culture container and were ready for cell seeding.

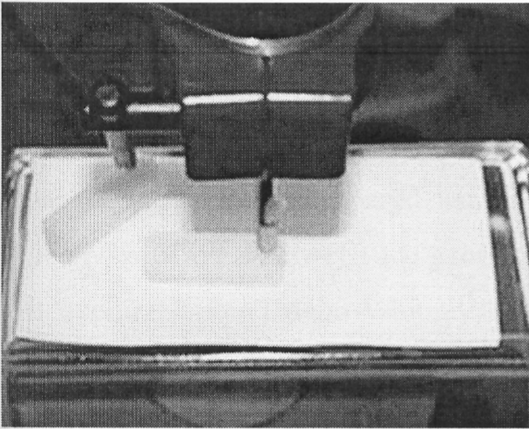
Figure 2- 16 Preparation of lyophilized ICBM



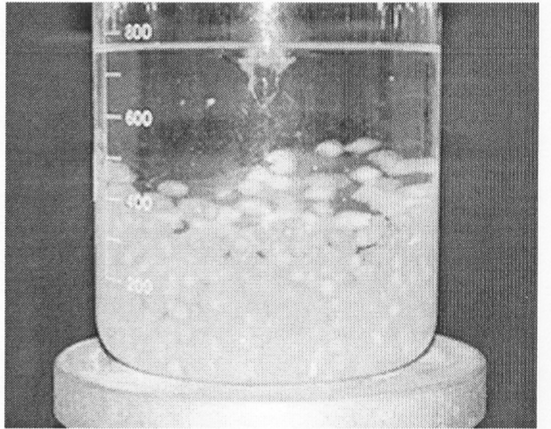
(a) Biochemical methods to remove fat, mineralization and protein in different solution



(b) ICBM size 12 mm in width and 10 mm in thickness

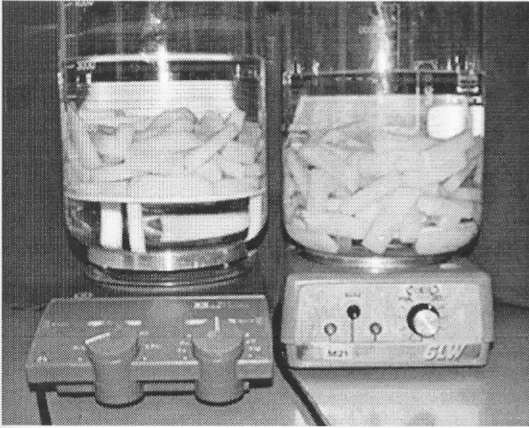


(c) Cutting ICBM into 5 x 3 mm size

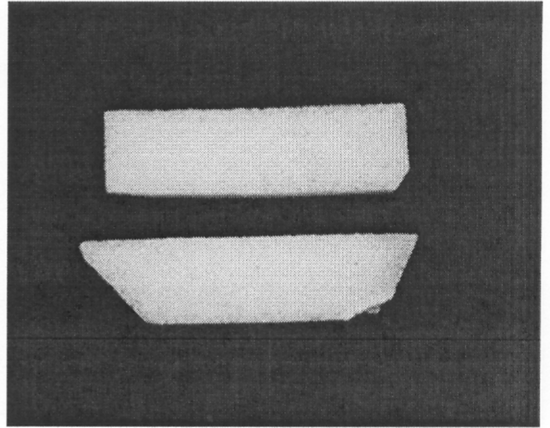


(d) Sterilization of ICBM scaffolds in chloroform and methanol 1:1 for 24 hours

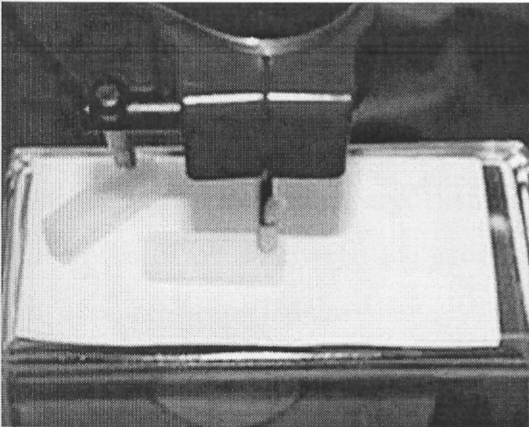
Figure 2- 16 Preparation of lyophilized ICBM



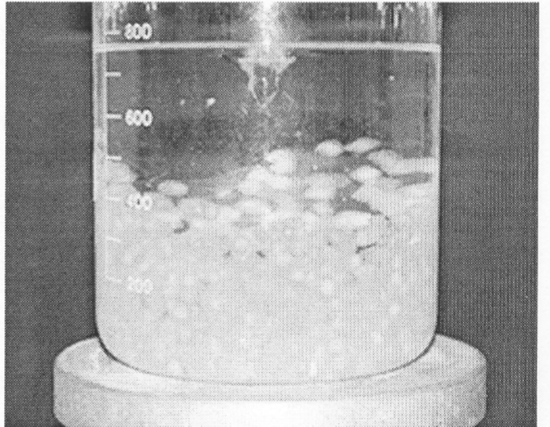
(a) Biochemical methods to remove fat, mineralization and protein in different solution



(b) ICBM size 12 mm in width and 10 mm in thickness



(c) Cutting ICBM into 5 x 3 mm size



(d) Sterilization of ICBM scaffolds in chloroform and methanol 1:1 for 24 hours

(e) Lyophilization of sterile ICBM scaffold using lyophilizator at -45°C for 12 hours

4. Statistics

4.1. Reliability control

A reliability control was performed in an analysis of densities of positive bands on gel elctrophoresis. Densities of bands on each gel were measured three times using NIH image software a public domain. Differences between measurements were compared using paired t-test at $p<0.05$. Average values of three measurements were used to create densities ratios between densities of observed parameters and house keeping gene, GDPH. The average values of two, three or five samples, according to designs of each study, were used in the analysis (Rosner, 1995b).

4.2. Parametric data

Parametric data were described using descriptive statistics and compared by using non-parametric staitistics. The data were analysed by 10-Student Stata for Windows (network), 1999. The differences of data between time points in the same group of study were compared using Wilcoxon signed-rank test, at $p<0.05$. The differences of data between the groups of study were compared using Two sample Wilcoxon rank-sum (Mann-Whitney) test, at $p<0.05$ (Rosner, 1995b).

These data included ALP activity, optical density of WST, concentration of calcium, concentration of TGF-β1, numbers of platelets and RBC, and the mineralization area.

4.3. Nonparametric data

The results were described and demonstrated by descriptive statistics (Rosner, 1995a). They are density ratios of gel electrophoresis