Diplomarbeit

In vitro cytocompatibility and gene expression of osteogenic markers in osteoblasts and growth plate chondrocytes after magnesium alloy treatment

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Abstract

Introduction: Nowadays fracture treatment in children is often done by surgical intervention. This tendency implies per se a high operation rate and an even higher one due to the, for this purpose mainly used, anticorrosive stabilisation items. This is the case because they have to be removed after fracture healing. The reoperation with all its troubles, risks and costs illustrates the advantages and also the necessity for biodegradable materials. Amongst the worldwide tested different degradable materials are also bioresorbable magnesium alloys. In this study two different alloys, ZX50 and WZ21 are tested in vitro in terms of their osteoinductivity and their cytocompatibility. Considering the alloys as possible materials for fracture stabilisation in paediatric surgery, more precisely in the growing bone, tests on an osteoblast cell line and on human growth plate chondrocytes are required.

Methods: For testing the osteoinductivity the MG63 cell line and the human growth plate chondrocytes were treated with the prior prepared eluates of the alloys. Treatment with pure SBF was to assess the influence of the vehicle while untreated cells served as control. After 48 hours mRNA of the samples was harvested and transcribed into cDNA. QRT-PCR was used to determine expression changes of the osteoinductive genes alkaline phosphatase and osteocalcin compared to the housekeeping gene GAPDH. To investigate cytocompatibility, namely metabolic activity and viability in different eluate concentrations, MTT and neutral red testing was performed. The MTT method is based on a conversion, the neutral red method on a colour up-take into the cells. The measurement of the optical density was conducted on a spectrometer at a wavelength of 550 nm and set into relation with the data of SBF and untreated cells, respectively.

Results: The 48 hours treatment of the hGPC with the ZX50 and the WZ21 eluate showed a significant alteration in both investigated genes. The treatment of the MG63 osteoblasts revealed an augmentation of alkaline phosphatase and osteocalcin only with the ZX50 eluate. The gene expression was compared to the levels of untreated and SBF treated cells. The cytocompatibility of the two eluates and of the two cell types diverged. While the WZ21 eluate neither influenced

viability nor metabolic activity throughout the tested concentration range, the ZX50 eluate treatment decreased viability and metabolic activity in both cell types. This decrease set in at a lower concentration for the hGPC than for the MG63 cells.
Discussion: Both tested alloys showed good cytocompatibility and an augmentation of osteoinductive markers. The ZX50 alloy appeared to be less cytocompatible than WZ21. This could be due to its faster degradation and the additional stronger increase in pH-number.
Key words: Magnesium alloys, biodegradable materials, trauma, epiphyseal growth plate.

Zusammenfassung

Einleitung: Der Trend der Knochenbruchversorgung im Kindesalter geht in den letzten Jahren stark in Richtung einer operativen Versorgung. Die aktuell verwendeten Nägel, Drähte, Schrauben und Platten bestehen überwiegend aus hoch korrosionsbeständigen Materialien. Diese Tatsache bringt die Notwendigkeit einer Reoperation zur Entfernung dieser nicht abbaubaren Teile, sowie alle mit der Operation einhergehenden Begleiterscheinungen, mit sich. Um nun eine zweite Intervention zu vermeiden wird weltweit intensiv an biologisch abbaubaren Materialien geforscht. In dieser Studie wurden die zwei verschieden zusammengesetzten biodegradierbaren Magnesiumlegierungen ZX50 und WZ21 in vitro auf ihre knocheninduzierende Wirkung sowie auf ihre Zytokompatibilität hin untersucht. Da das geplante Anwendungsgebiet dieser beiden Legierungen der noch im Wachstum befindliche kindliche Knochen sein soll, wurden alle Versuche sowohl an einer Osteoblastenzelllinie als auch an humanen Epiphysenfugenzellen durchgeführt.

Methodik: Zur Bestimmung der Osteoinduktivität wurden die beiden Zellarten, mit zuvor hergestellten Eluaten der beiden Implantate, inkubiert. Nach einer festgelegten Einwirkzeit von 48 Stunden wurde aus den Zellen mRNA isoliert und diese in cDNA umgeschrieben. Anschließend erfolgte mit Hilfe des Verfahrens der qRT-PCR die Untersuchung veränderter Level osteoinduktiver Marker im Vergleich zu unbehandelten, respektive mit SBF behandelten Zellen. Die hierbei untersuchten Marker waren alkalische Phosphatase und Osteocalcin. Als housekeeping-Gen wurde GAPDH verwendet. Zur Beurteilung der Zytokompatibilität wurden MTT und neutral rot Tests auf Basis von stofflicher Konversion beziehungsweise Farbaufnahme durchgeführt. Die Messung der optischen Dichte erfolgte mit einem Spectrometer bei einer Wellenlänge von 550 nm. Vergleichend wurde die optische Dichte SBF behandelter beziehungsweise unbehandelter Zellen herangezogen.

Ergebnisse: Die Level beider osteoinduktiver Marker zeigten, nach 48 stündiger Inkubation mit dem ZX50-Eluat, bei beiden Zellarten eine Erhöhung. Das WZ21-Eluat bewirkte nur bei den humanen Wachstumsfugenzellen eine gesteigerte Expression der alkalischen Phosphatase und des Osteocalcins. Die

Genexpressionslevel wurden immer in Relation zu unbehandelten, beziehungsweise SBF behandelten Zellen gesetzt. In der Zytokompatibilitätsprüfung ergab die Beimischung des WZ21-Eluates bei beiden Zellarten weder eine signifikante Reduktion der Viabilität noch der metabolischen Aktivität. Bezüglich der Wachstumsfugenzellen zeigte sich eine deutlich höhere Sensibilität gegenüber dem ZX50-Eluat verglichen mit der Osteoblastenzelllinie. Die Zelllinie zeigte erst ab einer Konzentration von 20 Volumsprozent einen Abfall der beiden Zytokompatibilitätsparameter.

Diskussion: Generell können beide Magnesiumlegierungen als zytokompatibel sowie osteoinduktiv erachtet werden. ZX50 zeigte etwas schlechtere Werte, welche möglicherweise durch die schnellere Degradierung und den damit verbundenen relativ starken pH-Wertanstieg zu erklären sind.

Schlagwörter: Magnesiumlegierungen, biodegradierbare Materialien, Trauma, Epiphysenfuge.

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Abbreviations

ALP	alkaline phosphatase
cDNA	complementary deoxyribonucleic acid
ECM	extracellular matrix
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAG	glycosaminoglycan
HA	hydroxyapatite
hGPC	human growth plate chondrocyte
HUVEC	human umbilical vein endothelial cell
I-CAM	intercellular adhesion molecule
IL	interleukin
κ-B-ligand (RANKL)	receptor for activation of nuclear factor
M-CSF	macrophage colony stimulating factor
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
MTT	dimethylthiazol-diphenyltetrezoliumbromid
nm	nanometre
NR	neutral red
PCR	polymerase chain reaction
PGA	poly-glycol-acid
PLLA	poly-L-lactic
qRT-PCR	quantitative real time polymerase chain reaction
REE	rear earth element
rpm	rounds per minute
SBF	simulated body fluid
TNSALP	tissue-nonspecific alkaline phosphatase

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1. Introduction

Bone fractures are common injuries in children. The general tendency away from conservative cast treatment towards surgical intervention with the advantage of early mobilisation is a fact. (Slongo 2002) Osteosynthetic materials like plates, wires, screws or elastic intramedullary nails are used for stabilisation. Till present days these items are mainly made out of highly anticorrosive materials, i.e. stainless steels, cobalt-chromium based alloys and titanium. (Staiger et al. 2006) So why is there a need to investigate in a rather new direction, namely biodegradable materials? Some good reasons can be given to underline the likeliness of having alternatives to the three above-mentioned. The first disadvantage of the commonly used anticorrosive materials is that they have to be removed from the body after a certain time period, to be precise after fracture healing. The second reason is the probably toxic abrasion or corrosion products of the materials, which can reach the human body's circulation. (Staiger et al. 2006) Thirdly, stress shielding due to unequal mechanical properties between bone and commonly used implants can occur. (Pietak et al. 2008a)

For these reasons it is very interesting to search for alternatives in the field of fracture surgery. The aim is to find a matter, which can be degraded by the body via non-toxic degradation products in a time period acceptable for bone healing. The minimal degradation time required guaranteeing sufficient fracture healing would be between 12 and 18 weeks. (Staiger et al. 2006, Witte et al. 2005) This is where the more than one hundred years ago started research on magnesium implants (re-)began. Magnesium has, in terms of osteosynthetic materials, some ideal and some less ideal characteristics. Therefore, it is compulsory to adjust and test different matters of this element in order to find a fitting composition for the mechanical, corrosive, biological and cytocompatible demands. The added elements should show no or at least a very low toxic effect on adjacent cells and the entire human body. Especially in paediatric surgery great importance should be attached to the latter.

Following the goal to find a suitable material, various researches on different

magnesium alloys were carried out. From these investigations it is known that implanted magnesium and its alloys stimulate the surrounding bone to form a stronger callus with increased bone mass. Besides, higher bone mineralization around the fracture side was observed. (Xin, Hu & Chu 2011, Witte et al. 2005, Kraus et al. 2012) This in turn raises the question of osteoinductive changes on the gene expression level of bone cells. Additionally, the levels of cytotoxic concentrations on the surrounding tissue are of interest.

Considering the growth plate as a thoroughly structured and precisely controlled system the necessity to test new magnesium alloys for children on osteoblast and on epiphyseal cells is highly obvious. Only with testing the epiphyseal cells the osteoinductive influence and the cytocompatibility of the magnesium alloys on the growth plate can be estimated. This diploma thesis deals with the osteoinductivity of the two alloys ZX50 and WZ21 on osteoblasts and epiphyseal cells and will give an insight view of in vitro gene expression changes of osteogenic markers. Moreover, the in vitro cytocompatibility of the two alloys considering the parameters metabolic activity and cell viability will be enlightened.

2. The bone

2.1 The bone in general

The bone is a main part of the musculoskeletal system, but it is also involved in the body's calcium and phosphate metabolism. In its latter function it provides homeostasis of the two mentioned and several other minerals. Mechanically, bone has to be weight carrying, absorbing external forces like compression, lengthening or tension and has to provide stiffness for appropriate muscle function at the same time. Certain bones host the red bone marrow and consecutively the production of blood cells, namely the haematopoiesis. The bone itself is surrounded by connective tissue. On its outer surface, except at cartilage-covered parts, it is encircled with the periosteum. The inner layer, which is very thin, is called endosteum. (Schiebler 1996, Seeman 2008, Tortora, Derrickson 2011)

Four different main types of bones can be distinguished:

long bones *(ossa longa)*
short bones *(ossa brevia)*
flat bones *(ossa plana)*
irregular bones *(ossa irregularia)*

Besides sesamoid *(ossa sesamoidea)*, pneumatic *(ossa pneumatica)* and accessory *(ossa accessoria)* bones have to be mentioned. (Weinberg, Hofmann & Claus 2006, Fanghänel et al. 2009) In this work the focus lies on longitudinal bone growth and consecutively on the long or hollow bones.

2.2 Macroscopic aspects of the bone

Macroscopically, a long bone can be divided in three different parts: diaphysis, metaphysis and epiphysis. These regions can be seen from the midpoint of embryonic development. **(see Figure 1)** (Shapiro 1987) The diaphysis mainly consists of a strong compacta, which surrounds the intramedullary space with its few trabecular structures. The furthermost part on both ends of a long bone is the epiphysis, which is entirely built out of cartilage at the beginning of bone development. The epiphysis is divided in three parts. It carries the articular cartilage that forms the joint. Adjacent to the articular cartilage lies the epiphyseal cartilage. In this area bone growth via a secondary ossification centre occurs. The third part is the growth plate also referred to as physis or epiphysis. **(see Figure 2)** (Forriol, Shapiro 2005) The metaphysis lies between the epiphysis and the diaphysis with its boundaries located between the growth plate and the space where the diaphysis broadens. The metaphysis together with the growth plate carries the groove of Ranvier. (Weinberg, Hofmann & Claus 2006, Forriol, Shapiro 2005)

Fig. 1 Macroscopic aspects of a long bone.

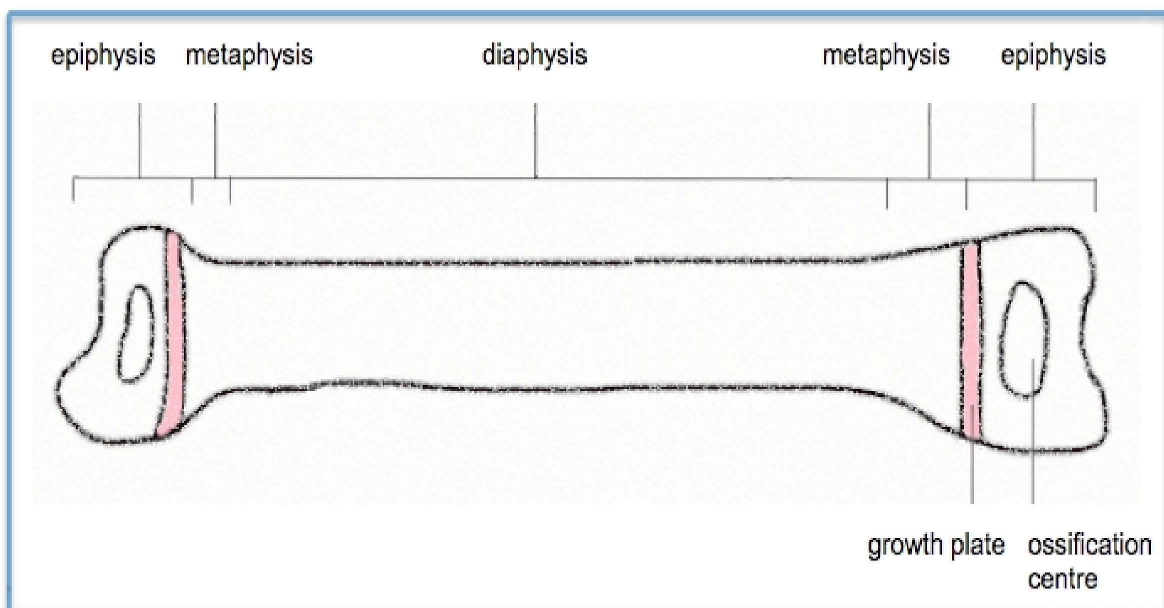


Fig. 2: Structural components of the epiphysis according to Forriol, Shapiro 2005:

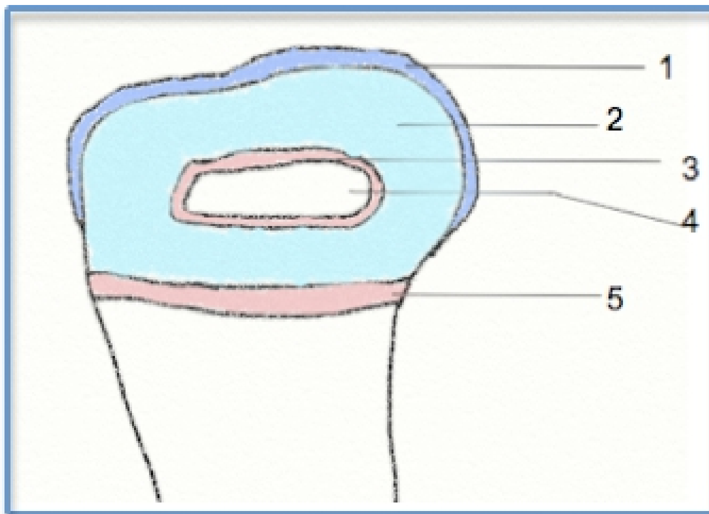
1) Articular cartilage

2) Epiphyseal cartilage

3) Growth plate of the secondary ossification centre

4) Secondary ossification centre

5) Growth plate



2.2.1 Bone substances

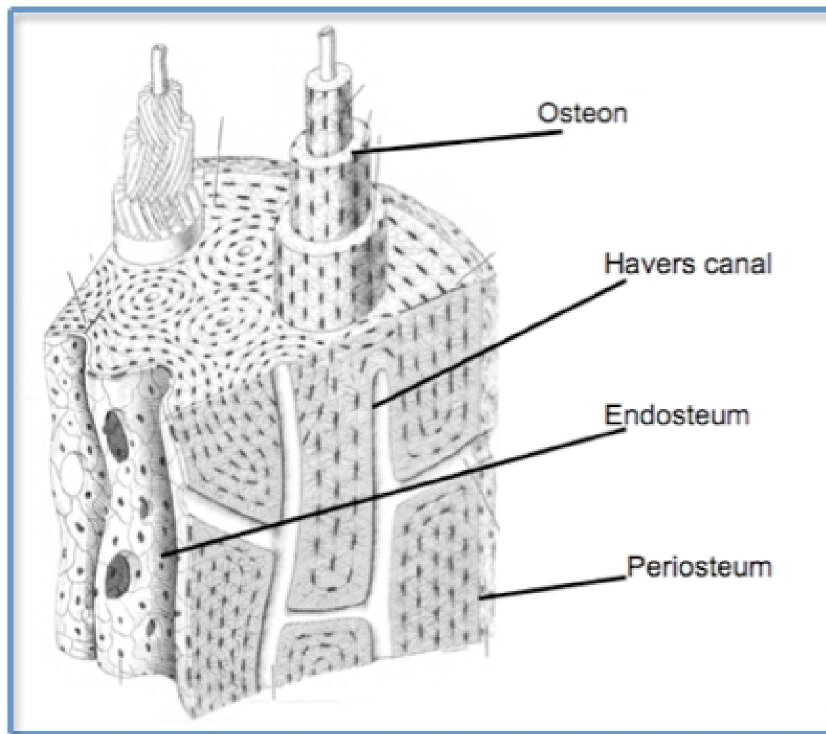
A bone is generally built out of the cortical or compact bone *(substantia compacta)* as firm surrounding structure and the trabecular or spongy bone *(substantia spongiosa)* placed within the bone. The spongy bone has a honeycomb like structure.

Compact bone:

The compact bone is located directly underneath the periosteum and represents the stress absorbing tissue. Built out of numerous, alike structural components called osteons or Haversian systems the tissue has a very high density and few rooms. An osteon is constructed out of concentric arranged lamellae around a blood, lymphatic vessels and nerves carrying canal. All osteons are unidirectional

and parallel to the length axis of the bone. (Tortora, Derrickson 2011) **(see Figure 3)**

Fig. 3: Schematic picture of a compact bone modified according to Schiebler 1996. (Schiebler 1996)



Spongy bone:

The spongy, trabecular or cancellous bone is always interior of a bone and lies under the compacta. The lamellae are of uneven structure and leave big spaces between them, which can be seen by the naked eye. This is to reduce the total weight of the bone. Although, firstly appearing unarranged, the trabeculae are well orientated along the lines of mechanical stress. Moreover, the cavities built up between the so-called trabeculae are filled with bone marrow. Depending on the age of the individual and the bone it is either red, blood producing bone marrow or yellow, fatty bone marrow. In case of great blood loss the yellow bone marrow, usually not involved in erythropoiesis, can rapidly change into red bone marrow. (Schiebler 1996, Tortora, Derrickson 2011)

2.3 Microscopic aspects of the bone

Microscopically, the bone consists of cellular components and the extracellular matrix. The latter represents the bigger part of the total bone mass.

The cellular components are: Osteoprogenitor cells
Osteoblasts
Osteocytes
Osteoclasts

Osteoprogenitor cell:

The osteoprogenitor cells are differentiated out of the pluripotent mesenchymal stem cells through various precursor and progenitor stages to osteoblasts respectively to osteocytes. Furthermore, osteoprogenitor cells are responsible for bone growth and have a lifelong involvement in fracture healing. (Weinberg, Hofmann & Claus 2006, Klein-Nulend, Bonewald 2008)

Osteoblast:

The main assignment of osteoblasts is the production and excretion of the organic components of the extracellular matrix, like collagen, glycoproteins and proteoglycans. In their terminal differentiation they become osteocytes, get into a resting state or undergo apoptosis. (Gratzl 2005)

Osteocyte:

The osteocyte, which is immured in the calcified bone, is the most abundant cell type of the bone. It has a ten fold higher occurrence than osteoblasts. Osteocytes show numerous vertically erected processes, through which they are connected via gap junctions to each other and to the bone surface. They seem to be responsible for mechanical induced bone remodelling. (Schiebler 1996, Klein-Nulend, Bonewald 2008)

Osteoclast:

The bone resorbing osteoclasts develop from the hematopoietic cells and belong to the monocyte-macrophage lineage. According to that, they are huge cells with

numerous nuclei. Their formation depends on the receptor for activation of nuclear factor κ-B- ligand (RANKL) and the macrophage colony stimulating factor (M-CSF). The function of the osteoclast is to resorb calcified cartilage, mineralized bone and dentine, if required. (Weinberg, Hofmann & Claus 2006, Takahashi et al. 2008)

2.4 Extracellular matrix

The composition of the extracellular matrix (ECM) shows approximately:

55 % inorganic minerals

30 % organic compounds

15 % water (Tortora, Derrickson 2011)

2.4.1 Inorganic minerals

Phosphate and calcium are by far the predominant elements of the inorganic minerals of the bone. The rest of the inorganic minerals, about eight percent, are nitrates, magnesium, sodium, fluorine and others. The mineral salts are prevalent as calcium phosphate ($Ca_3(PO_4)_2$) and calcium hydroxide ($Ca(OH)_2$). They form calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and other calciumphosphate crystals. The so-called osteoid, which is synthesized by osteoblasts, is primarily not arranged in the typical hexagonal crystalline structure of the hydroxyapatite (HA). Forming the crystals requires the presence of collagen fibres. The tiny spaces between the collagen acts as a starting point for crystallization. This special structural organisation of the minerals gives rise to good compression strength of the bone. (Schiebler 1996, Speckmann, Hescheler & Köhling 2008)

2.4.2 Organic compounds

Amongst all organic compounds a collagen content of 90 percent can be found. The highly prevalent protein collagen with its 28 different types is very important for strengthening the bone in terms of tractive forces. (Speckmann, Hescheler & Köhling 2008) In the group of the collagens, collagen type I is the predominant form. (Schiebler 1996, Bou-Gharios, de Combrugghe 2008) Collagen I, and several of the other collagen types, are fibrillar collagens, meaning that they consist of three long continuous fibrils, which determine their mechanical characteristics. The rest of the organic mass consists of proteoglycans and glycoproteins. Even if

these matrix proteins are rare, importance must be attached to them. (Bou-Gharios, de Combrugghe 2008)

Proteoglycan:

The proteoglycans are characterized by long polysaccharides, namely glycosaminoglycans (GAGs), which are connected to a protein centre. Important representatives of this large sized molecule family are aggrecan, verican, decorin and biglycan. (Robey 2008)

Glycoprotein:

Glycoproteins are, like proteoglycans, a linkage between a protein and a saccharide but with a higher protein fraction. The earliest detected protein of this class was osteonectin. It can potentially bind Ca^{2+} and collagen or act as starting nucleus for HA formation. An osteonectin deficiency causes osteopenia with decreased size of hydroxyapatite crystals. The sialoprotein osteopontin is mainly produced at the expiry of osteoblast formation just before starting the bone's mineralisation. A deficiency of osteopontin causes an increased mineral content of the bone while producing smaller HA crystals. (Robey 2008)

The alkaline phosphatase (ALP) appears anywhere in the human body and in nature. In the body it exists in four isoenzymes of which three are specifically related to the intestine, the mature placenta and the germ cells, respectively. The fourth is called tissue-nonspecific ALP (TNSALP) and can largely be found in the early placenta, the liver, the kidney and the bone. The ALP catalyses different compounds within a special pH value. For every catalytic activity of the enzyme Mg^{2+} is required. (Whyte 2008, Cremers, Garnero, Seibel 2008) With the aid of alkaline phosphatase, osteoblasts split off pyrophosphat to allow mineralization of the matrix. (Schmidt, Lang & Heckmann 2010)

2.5 Histologic bone types

Although, composed out of the same elements bone shows two different structural patterns under the microscope. This is why a histological distinction between woven and lamellar bone is made.

Woven bone:

Woven bone is characterised by an irregular construction of collagen fibres and the extracellular matrix. No unidirectional lamellae are visible. Every developing bone goes through the stadium of woven bone, which has a lower mineral fraction than lamellar bone. (Schiebler 1996)

Lamellar bone:

The lamellar bone forms the compact and trabecular bone of an adult except in case of fast remodelling and fracture repair. It is built out of parallel-organised collagen fibre bundles. Each of these bundles has a Haversian or central canal that carries the neurovascular supply. The collagen lamellae are precisely arranged around this canal. The recurring structural unit of lamellae and Haversian canal is called Haversian system or osteon. The osteons lie very close and parallel to each other, directed along the long axis of the bone. (Wigley 2008)

2.6 Bone development

The bone develops either directly out of mesenchymal stem cells (MSC) or indirect via cartilage as a preliminary template, which gets replaced by bone over time. The direct method is called intramembranous ossification, while the indirect one is called endochondral ossification. Both ways, though developing differently, do not show structural dissimilarities of the mature bone. In the two cases the prior built bone is immature and changes into mature trabecular bone. (Schiebler 1996, Tortora, Derrickson 2011, van der Eerden, Karperien & Wit 2003)

2.6.1 Intramembranous ossification

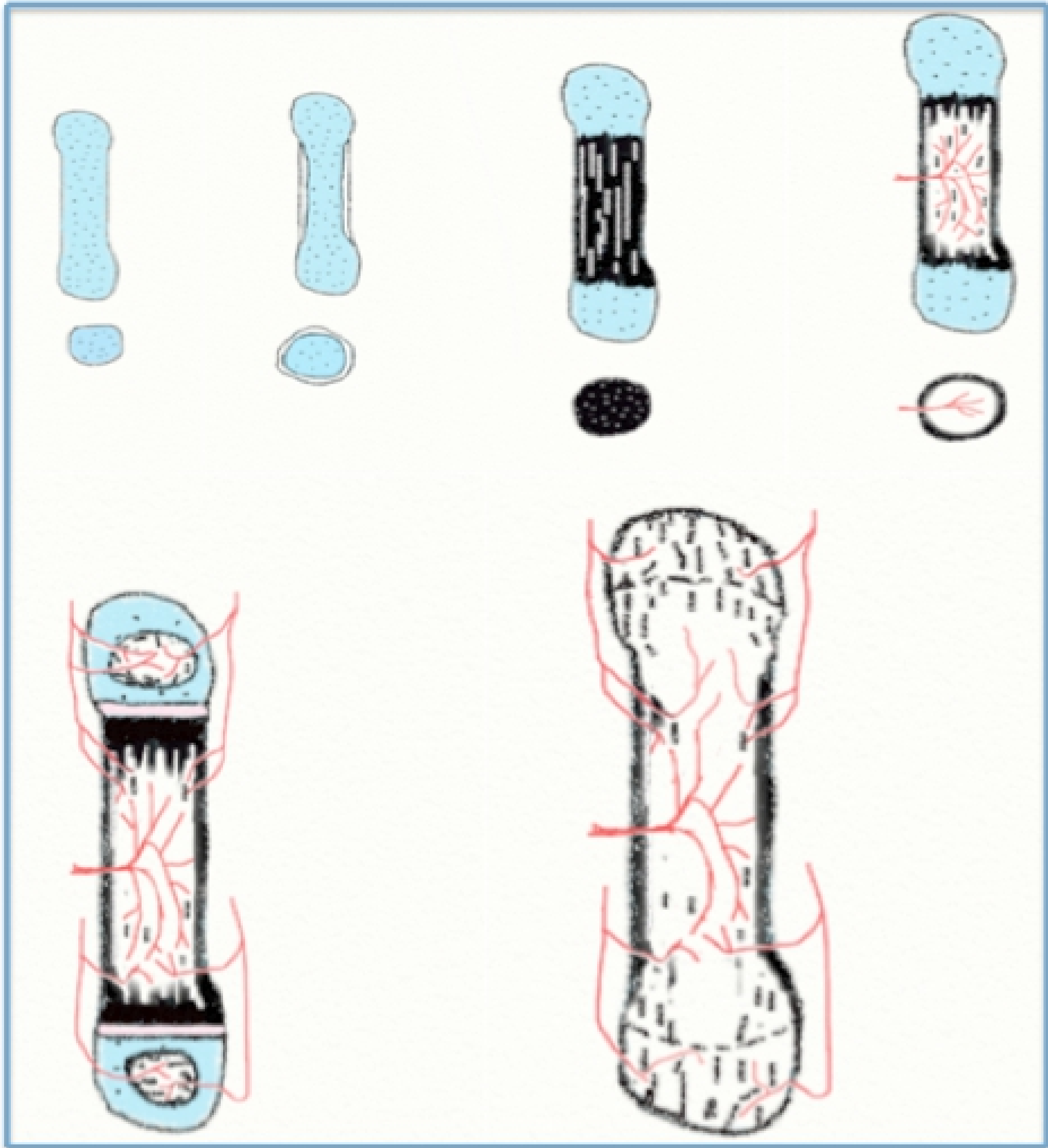
The intramembranous ossification starts with the formation of a primary ossification centre at the location where the bone will develop. This is conducted by the condensation of MSC. The condensation happens around the sixth week of pregnancy and at that time the first models of hyaline cartilage are formed. (Sadler 2003) First the stem cells are transformed into osteoprogenitor cells and later to osteoblasts. The osteoblasts synthesize still unaligned collagen and osteoid, namely the extracellular matrix. Soon after that, the osteoblast is now called osteocyte and mineralization through precipitation of calcium and phosphate sets in. Finally, the different primary ossification centres fuse into one bone structure - the woven bone or primary spongiosa. Around these early, thin trabeculae more extracellular matrix is produced and calcification progresses, so the structures solidify. In later spongy bone, spaces get filled with bone marrow while in the future compact bone trabeculae thicken constantly.

Except from some facial bones and parts of them, the bones of the skeleton are mostly not built solely via intramembranous ossification. (Schiebler 1996, van der Eerden, Karperien & Wit 2003, Tortora, Derrickson 2011, Wigley 2008)

2.6.2 Endochondral ossification

The principal of the endochondral bone formation is the replacement of a pre-shaped cartilage into bone. This type of ossification is typical for long and short bones. It is the most common way of bone formation. (Schiebler 1996)
Out of mesenchymal cells chondroblasts develop and gather. This takes place at the end of the embryonic period between the seventh and eighth week of pregnancy. (Sadler 2003) In the following, a hyaline cartilage is formed and with the cessation of extracellular matrix excretion the chondroblasts become chondrocytes. The growth of the cartilage is either carried out endogenous, by cell division or appositional, by accumulation of the ECM. In the course of cartilage growth some chondrocytes located in the middle get hypertrophic. Some other chondrocytes undergo apoptosis due to insufficient nutrition caused by on-going calcification of the mid-region. This is where osteoblasts develop and the primary ossification centre is formed near a nutrient artery in the diaphysis. The built bone is a trabecular bone and grows in the direction of the epiphysis. Most of the trabeculae are replaced either by bone marrow or by compact bone. As the articular artery enters the epiphysis the second ossification centre is formed similar to the above-described way. The leftover cartilage between the epiphysis and the diaphysis builds the epiphyseal growth plate. (Tortora, Derrickson 2011) **(see Figure 4)**

Fig. 4: Simplified illustration of endochondral ossification modified according to Schiebler 1996. The round structures under the first line show a cross-section of the corresponding upper bone. (Schiebler 1996)



2.7 Bone growth

Bone growth during infancy, childhood and adolescence can also happen in the two above-described different ways: via intramembranous and via endochondral ossification.
The elongation happens endochondrically. In that case the bone extends due to apposition of bone material at the diaphyseal end of the physis. More concrete: an interstitially, from the epiphyseal towards the diaphyseal side, growing hyaline cartilage gets mineralized. (Karaplis 2008, Tortora, Derrickson 2011)

The circumferential growth or growth in thickness of the diaphysis, and to some extends the metaphysis, is conducted intramembranously. For this purpose the groove of Ranvier is necessary. The circumferential growth is carried out via direct apposition of osteoblasts from the periosteal inner cambial layer. These osteoblasts mainly produce collagen type I and other organic molecules. Out of these materials the extracellular matrix and later the new concentric lamellae are formed. As the extracellular matrix embeds the osteoblasts they develop into osteocytes. On the other side, to maintain proportions between bone cavity and cortical layer, osteoclasts are resorbing bone from the inner endosteal surface. Meanwhile, the compacta gets thicker, the bone cavity broadens. (Forriol, Shapiro 2005, Tortora, Derrickson 2011)

2.7.1 The growth plate (physis, epiphysis)

The cartilaginous structure growth plate is located between the epiphysis and the metaphysis. In the bigger long bones one growth plate at each end of the bone can be found, while in the shorter bones just one physis is present. (Sadler 2003)
The physis mainly consists of chondrocyte layers in different stages of differentiation and maturation. It is divided in four different zones of a highly organised micromorphologic structure. From the epiphyseal to the metaphyseal side the naming of the zones diverges, though describing the same pattern:

Germinal, resting, reserve or stem cell zone

Column, transition or proliferation zone

Hypertrophic zone

Ossification zone **(see Figure 5)** (Forriol, Shapiro 2005, van der Eerden, Karperien & Wit 2003)

Germinal zone:

The germinal cell zone consists of randomly spread stem cells in a huge amount of extracellular matrix, collagen and proteoglycans. Although, being relatively inactive, the area shows in some parts mitotic activity with consecutive release of cells into the proliferative zone. This release is guided by a still unknown mechanism. (Karaplis 2008, Ballock, O'Keefe 2003)

Column zone:

The down streaming signals and the habit of a vertically directed mitosis of the chondrocytes, in the upper zone of the column zone, make the cells align in fairly regular columns. The columns are separated by longitudinal septae. In the upper zone, also referred to as proliferation zone, cells are in a flat shape. The lower zone of the column zone is the maturation zone. As the name of the two subsections hints, after a certain time of cell division and extracellular matrix protein production, cells start to proliferate and become larger in size. The excreted matrix proteins are besides proteoglycans mainly collagen type-II, -IX and -XI. (Ballock, O'Keefe 2003, van der Eerden, Karperien & Wit 2003)

Hypertrophic zone:

In the hypertrophic zone the chondrocytes go trough their terminal differentiation. They become very large and round in size with the main purpose in secreting type-X collagen, which is uniquely found in the hypertrophic zone of the epiphyseal growth plate. Additionally, the alkaline phosphatase activity increases. (van der Eerden, Karperien & Wit 2003) The matrix in this zone is mainly not mineralized but starts to be so in the area adjacent to the metaphysis. Matrix vesicles placed within the extracellular matrix function as starting points for the mineralization. (Ballock, O'Keefe 2003) In the lower parts of the zone the hypertrophic

chondrocytes are up to five times the size of a usual chondrocyte. They undergo apoptosis leaving a scaffold of extracellular matrix. (Karaplis 2008)

Ossification zone:

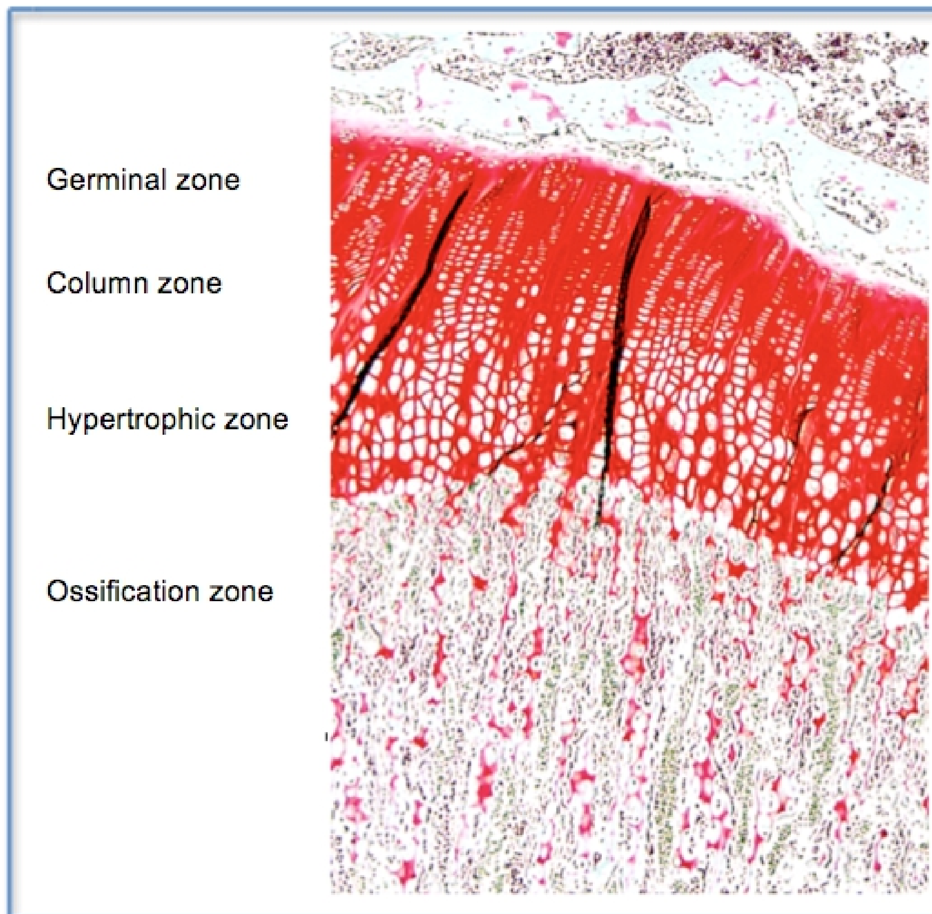
In this area two thirds of the longitudinal septae, which formally divided the different columns, are resorbed by osteoclasts. Along the remaining one third and the above-mentioned ECM template, osteoblasts settle and start to produce bone matrix. (Ballock, O'Keefe 2003) After the mineralization of the latter, woven bone emerges. Most of the woven bone is resorbed by osteoclasts and in the following replaced by trabecular bone. (Schiebler 1996) To ensure nutrition of this area an ingrowth of capillaries from the metaphyseal side is induced. The inductors of the vascularisation are a low oxygen tension and the excretion of vessel endothelial growth factor (VEGF). (Forriol, Shapiro 2005, Karaplis 2008, Schneidmüller, Weinberg 2006, van der Eerden, Karperien & Wit 2003)

2.7.2 The perichondrial ossification groove

The perichondrial ossification groove consists of the wedge-formed groove of Ranvier and the fibrous ring of LaCroix. The groove of Ranvier surrounds the resting zone and consists of chondrocyte progenitor cells. Its function is to allow expansion of the growth plate in width as the bone grows in length.

The ring of LaCroix gives mechanical support to the growth plate. The band-like shaped structure is connected to the periosteum. (Ballock, O'Keefe 2003, Burdan et al. 2009, Forriol, Shapiro 2005)

Fig. 5: Epiphyseal growth plate of a 4 weeks old Sprague-Dawley rat, Safranin-O stained, 5 µm thick section, magnification 5 times.



3. Fractures in children

3.1 General aspects of fractures in children

The Müller-AO-classification usually used for division of fractures in adulthood is mostly inapplicable on children. This is due to special features and structures of the juvenile bone. At the growth plate for example, an area of lower resistance against mechanical forces, the appearance of the injury may be similar despite different traumata causing the lesion. The higher flexibility, which is due to the lower mineral density of children compared to adults' bone, also influences the fracture forms. Moreover the young bone is able to absorb more energy from an impact and its cortex is better protected by a relatively thick periosteum. (Currey, Brear & Zioupos 1996, Schneidmüller, Weinberg 2006, Randsborg, Sivertsen 2012)
For fractures in the growing skeleton the main differentiation is made between articular and non-articular fractures. Concerning the latter, fractures of the metaphysis are widely included.

Due to the above-mentioned special mechanical properties of the juvenile bone, there are some noteworthy fractures occurring exclusively in children. At this, for instance at the greenstick fracture, the buckle fracture and the epiphyseal fractures have to be mentioned together with the complete or adult type fracture. Another child specific injury is the transitional fracture, affecting the partly closed growth plate. The therapeutic intervention is depending on the age of the child, location, distance to the physis and degree of dislocation. (Schneidmüller, Weinberg 2006, Randsborg, Sivertsen 2012)

3.2 Fracture types occurring in children

3.2.1 Non-epiphyseal fractures

Greenstick fracture:

The greenstick fracture can be located either in the metaphysis or in the diaphysis of a long bone. The trauma causes an interruption of the corticalis just on the tension but not on the compression side of the bone. This unilateral discontinuity of the corticalis can be seen on x-ray pictures. In most cases the periosteum around a greenstick fracture stays unimpaired. For that reason, this type of injury is considered to be slightly unstable especially within the first two weeks after the injury took place. (Flocken, Bassir & Freyschmidt 2006, Niethard, Pfeil & Biberthaler 2009, Randsborg, Sivertsen 2012)

Buckle fracture:

The buckle fracture materializes only in the metaphysis of the bone and occurs mainly due to compression along the vertical axis. On the x-ray pictures an outward bulge or a notch but no fracture line trough the corticalis is visible. A straightforward fracture healing can be expected since buckle fractures are considered to be stable. (Flocken, Bassir & Freyschmidt 2006)

Bowing fracture:

The bowing fracture happens because of bending forces on a long bone. No corticalis alterations but a plastic deformation of the bone can be seen on the x-ray pictures. (Flocken, Bassir & Freyschmidt 2006)

Toddler's fracture:

The toddler's fracture is typical for infants who are still unsafely walking or running. It is a spiral fracture located at the tibia that often materializes without a direct trauma noticed. X-ray diagnosis can be tricky but fracture healing usually conducts without problems. (Flocken, Bassir & Freyschmidt 2006)

Avulsion fracture:

If one compares the toughness of the ligaments and tendons proportionally to the bone strength in children, it becomes clear that the bone is much weaker and avulsion type fractures occur frequently. This difference in strength explains why the bone breaks before the ligaments or tendons rupture. Avulsion fractures are located at the area of insertion of a tendon or a ligament. The fracture line can be seen at a right angle to the tension force causing the injury. (Niethard, Pfeil & Biberthaler 2009)

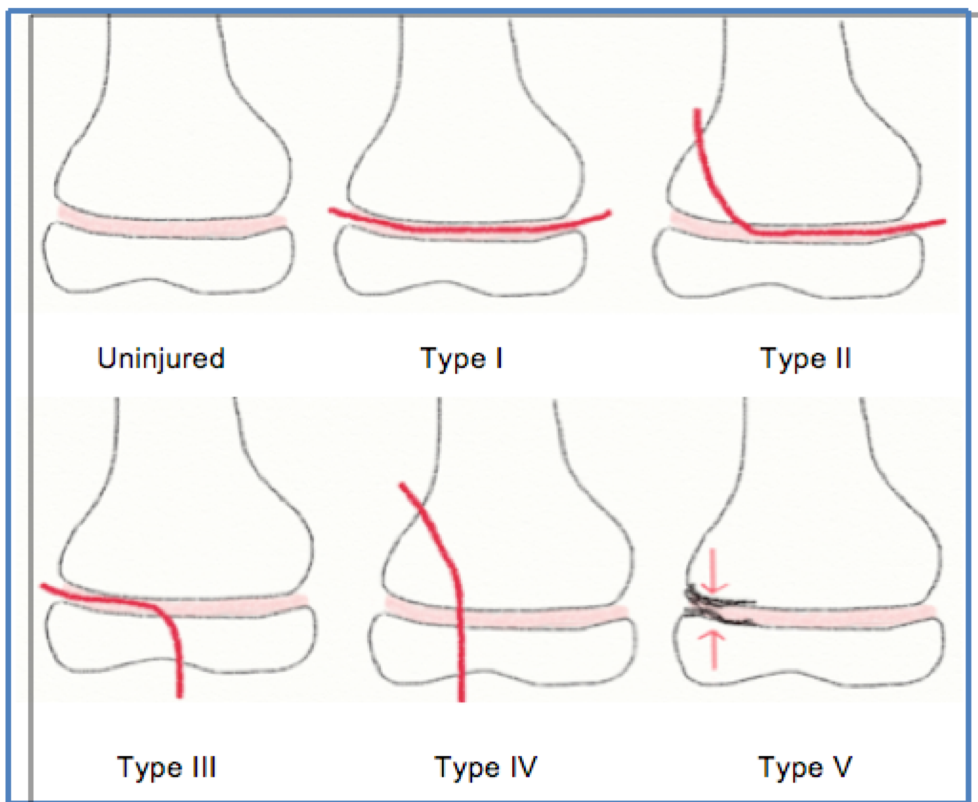
3.2.2 Epiphyseal fractures:

15 percent of all fractures in children affect the epiphysis. (Slongo 2002) These lesions are classified according to Salter-Harris in five types. **(see Figure 6)** (Niethard, Pfeil & Biberthaler 2009)

Salter-Harris classification:

- Type I: Is a fracture that splits the epiphysis from the metaphysis directly and uniquely trough the growth plate.
- Type II: The fractures line goes through a part of the physis and splits of a metaphyseal wedge.
- Type III: The fracture partly separates the epiphysis from the metaphysis and splits off an epiphyseal wedge.
- Type IV: This fracture affects the epiphysis and metaphysis while running orthogonally to and through the growth plate.
- Type V: The mechanism leading to this fracture is a compressive force along the long axis of the bone, thus the physis gets crushed. (Mayr 2006)

Fig. 6: Salter-Harris fracture types according to Niethard, Pfeil and Biberthaler 2009. (Niethard, Pfeil & Biberthaler 2009)



2.2.3 Transitional fracture

Transitional fractures are injuries that can happen to the growth plate when the physis is already partially closed. It is a typical injury for the adolescent skeleton. In this case the not totally closed growth plate breaks until it reaches the already ossified area. At that point, the fracture line is redirected either to the joint or in orientation to the metaphysis. These fractures are classified as twoplane- or triplane-fractures. (Schneidmüller, Weinberg 2006, Ruffing, Muhm & Winkler 2011)

3.3 Fracture healing

Fracture healing is a very complex and well-orchestrated physiologic process. The remarkable thing about fracture healing is that, respective of the individuals age, pre-fracture conditions can be restored. This means that a bony fracture can heal without a fibrous scare and can be reconstructed to former biomechanical circumstances. This requires repeating some features of embryologic skeletal development. (Gerstenfeld et al. 2003, Giannoudis, Einhorn & Marsh 2007) Fracture healing is conducted via endochondral and intramembranous ossification. (Mark et al. 2004, Marsell, Einhorn 2011, Niethard, Pfeil & Biberthaler 2009, Schiebler 1996)

Generally a bone fracture can histologically recover in two different ways: via direct and via indirect fracture healing.

3.3.1 Direct fracture healing

Direct fracture healing distinguishes between contact and gap healing. Both types usually do not occur naturally but do so after surgical fracture stabilisation. Thus, intrafragmentary movement is prevented and gaps between fracture sides can be minimalized, direct remodelling of the lamellar bone and the Haversian canals is achieved. (Giannoudis, Einhorn & Marsh 2007) For contact healing an interfragmentary gap of less than 0,01 mm is required. Over that distance cutting cones out of osteoclasts and osteoblasts directly bridge the fracture cleft. The osteons are remodelled straight into lamellar bone without any callus formation. In case of gap healing the gap must be less than 0,8 mm. At this the bony reunion and the lamellar remodelling do not happen simultaneously but one after another. (Marsell, Einhorn 2011)

3.3.2 Indirect fracture healing

The indirect or secondary fracture healing represents the major fraction of bone repair. (Giannoudis, Einhorn & Marsh 2007) It is conducted via both endochondral and intramembranous bone healing. For this healing type neither totally stable conditions nor rather small interfragmentary gaps are compulsory. (Marsell, Einhorn 2011)

The indirect fracture healing can be divided in four phases:

1. The first phase includes the initial injury with an acute inflammatory response, which is necessary for the coagulation of the hematoma. In the following, the initiation of angiogenesis and the chemotaxis of inflammatory and mesenchymal stem cells (MSC) occur. Involved proinflammatory signals are tumour necrosis factor-α (TNF-α) and the interleukins 1, 6, 11 and 18.
2. The subsequent endochondral formation of a cartilaginous callus out of the hematoma requires the MSC, which where recruited from the surrounding soft tissue, the bone marrow, the periosteum and the pool of peripheral stem cells from the blood flow. Around the 7th to 9th day post injury the soft callus is at its peak while simultaneously the intramembranous formation of a hard callus, starting directly at the fracture side, occurs. In the following, the vascular in-growth is induced either via an angiopoietin-dependent or the vascular endothelial growth factor (VEGF)-dependent pathway. The VEGF-pathway is considered to be more essential.
3. In this stage the callus' chondrocytes undergo proliferation, hypertrophy and the ECM becomes calcified. Ensuing the resorption of the calcified cartilage and the formation of woven bone, with the deposition of calcium and phosphate, takes place. Calcium and phosphate later become the side of origin of apatite crystallisation. This succession replays the embryological bone development. The peak of the hard callus formation can be measured with the ECM markers procollagen, alkaline phosphatase, osteocalcin and osteonectin.
4. To establish lamellar bone osteoclasts and osteoblasts remodel the hard

callus and set up the bone cavity. This process can last for years till pre-fracture conditions are achieved. In animals and children a shorter time period for finishing this process can be estimated. (Gerstenfeld et al. 2003, Marsell, Einhorn 2011)

3.4 Fracture fixation in children

The general main goals of fracture treatment in children are to achieve stabilisation, anatomic reposition and preservation of the physis. Moreover, pain reduction and protection of the surrounding soft tissue can be attained. (Skutek, Krettek 2006) At this a decision between a conservative cast treatment and a surgical intervention must be made. Cast treatment or other stabilizing bandages are used frequently in rarely dislocated and stabile fractures for four to six weeks. (Niethard, Pfeil & Biberthaler 2009) From the operative viewpoint, fracture stabilisation can be carried out with Kirschner-wires, elastic stable intramedullary nails (ESIN), screws, submuscular plates, and locked nails. (Baldwin et al. 2011, Niethard, Pfeil & Biberthaler 2009)

Elastic stable intramedullary nails:

The elastic stable intramedullary nailing is a closed surgical intervention for long bone fracture treatment in children. It was used for femur fractures and forearm fractures first. Mostly two nails are used, pre-curved and brought into the medullar cavity by a small hole in the metaphysis. Two indistinguishable bowed nails brought in abreast, one from lateral and the other one from medial, are sufficient to stabilise the fracture. (Ligier et al. 1988)

4. Bioresorbable implants - an alternative to commonly used implants

Since the tendency of facture treatment in children in the last decades is shifting from conservative to surgical intervention, researchers have started to look for alternatives to highly anticorrosive materials such as titanium, stainless steels or cobalt-chromium based alloys. (Slongo 2002, Staiger et al. 2006) The mentioned, though nowadays used throughout for fracture stabilisation, do have the great disadvantage of not being degradable in biological environments. This characteristic feature implies the need for removal of the stabilizing items. The reoperation for taking out the implant involves additional pain, a second hospital stay and a repeated narcosis. This causes a higher health risk for the patient due to the operation in general and the side effects of the anaesthetics.

In paediatric surgery hardly any resorbable materials, except pins for refixation of osteochondral fractures and plates in craniofascial surgery, are in clinical use. This is even though, due to extremely short healing time in children, lower mechanical material performance would be required. (Slongo 2002)

In the past decades different materials for this purpose have been tried out. At this, metallic materials like iron or magnesium based alloys have to be named. (Staiger et al. 2006) Moreover, polymers out of α-hydroxyacid like poly-L-lactic (PLLA) and poly-glycol-acid (PGA) and other polypeptides were under investigation. (Vert et al. 1998)

4.1 History of the magnesium alloys

In 1878 the first use of magnesium as biodegradable material for surgical intervention had been documented. It served as suture material for a ligature of a bleeding human vessel. In 1892 the Austrian physician Erwin Payr explored various clinical applications for magnesium as a degradable material like tubes for anastomosis, plates, arrows, wires and rods. Research from different people at the time around 1900 shows some still valid results: the magnesium degradation time is influenced by the size and thickness of the object and the blood flow in the treated organ. It has antithrombotic effects as a connector in vessel anastomosis and shows an "overproduction of callus" if used in osteosynthetic purpose. Despite enormous gas formation the magnesium does not cause visible harming to the nearby tissue. (Witte 2010)

4.2 Magnesium alloys: state of knowledge

In the past two decades the research on biodegradable magnesium alloys experienced a great revival. What, however, makes magnesium so interesting and promising for vessel stenting and osteosynthetic materials? First of all, it is a naturally existent essential lightweight metal in the human body. It has a density of 1,73 g/cm^3 (Pietak et al. 2008b) and can be found in great amounts in the bone tissue. (Persaud-Sharma, McGoron 2012, Purnama et al. 2010, Staiger et al. 2006) Basically magnesium metal shares its mechanical features, like density, strength and Young's modulus with the human bone. For that it can be load carrying and should not cause stress shielding like it occurs in titanium alloy and stainless steel treatments. (Ye et al. 2010, Huan et al. 2010, Staiger et al. 2006) Better callus formation (Witte 2010) , a significant higher mineralization rate and periosteal bone growth nearby magnesium implants in vivo have been observed, while the adjacent soft tissue showed no signs of mineralization or bone formation. (Witte et al. 2005) This indicates that magnesium has a local osteoinductive effect on the bone tissue, namely the osteoblasts. These characteristics are obviously very favourable for fracture surgery items. (Witte et al. 2005, Staiger et al. 2006, Yamasaki et al. 2003)

Additionally, magnesium dissolves and disappears completely in a liquid surrounding, which is a great advantage compared to common osteosynthetic materials. So a second surgical intervention to remove the material after fracture healing can be avoided.
In terms of influence of magnesium on the entire body some facts can be pointed out. It could be shown that in an individual with unrestrained kidney function hypermagnesaemia is very unlikely. This is because of the magnesium's potent excretion in the urine. (Staiger et al. 2006) It was further demonstrated, that a magnesium alloy pin, degrading within 90 days in a rabbit femoral shaft, did not increase serum magnesium levels of the animals. The normal required daily magnesium intake for and adult human person lies between 300 and 400 mg. (Purnama et al. 2010)

Were does magnesium set a problem?
In terms of mechanical performance pure magnesium shows a low elastic module and is consecutively rather brittle. (Witte 2010) Another problem is its fast degradation via corrosion in electrolytic fluids especially in the presence of chloride ions. (Ye et al. 2010, Persaud-Sharma, McGoron 2012, Staiger et al. 2006) According to that and its above-mentioned mechanical characteristics, magnesium in its pure form does not provide an appropriate weight bearing performance over time. The corrosion time of the item depends on the medium in which the material is solved. More precisely, the degradation is influenced by protein and amino acid contends as well as inorganic components of the fluid. (Xin, Hu & Chu 2011) Despite different compositions of the dissolving fluid in vitro, degradation is always accompanied with strong gas evolution due to oxidation-reduction reactions. These gas evolutions were also seen on x-ray pictures of in vivo tests with different magnesium alloys. (Persaud-Sharma, McGoron 2012, Staiger et al. 2006, Kraus et al. 2012)
The net reaction (1) of magnesium in an aqueous surrounding as well as the oxidation (2) and the reduction (3) reaction are given below. The abbreviations (s), (aq) and (g) stand for the solid, aqueous and gaseous aggregate phase.

1) $Mg\ (s) + 2H_2O\ (aq) \rightarrow Mg\ (OH)_2\ (s) + H_2O\ (g)$
2) $Mg\ (s) \rightarrow Mg^{2+}\ (aq) + 2e^-$
3) $2H_2O\ (aq) + 2e^- \rightarrow 2H_2\ (g) + 2OH^-\ (aq)$ (Purnama et al. 2010)

Every mole of magnesium released from the implant produces, as the equation demonstrates, one mole of hydrogen gas. (Hanzi et al. 2010) The gas forming causes problems especially in orthopaedic applications. If used intravascularly, the gas is effortlessly transported away because of the higher blood flow in the vessel. Despite everything, it should be considered that standard magnesium alloys commonly lack purity, for that all results should be interpreted critically. (Feyerabend et al. 2010, Hort et al. 2010)

To overcome the problems the magnesium entails, different methods like coating, dicalciumphosphate dehydration, alkali heat treatment and alloying have been

tried out. (Purnama et al. 2010) Calcium, zinc, manganese, aluminium and rear earth elements like yttrium, gadolinium and zirconium, including others were investigated as composing materials with magnesium. (Ye et al. 2010, Huan et al. 2010, Li et al. 2008, Hanzi et al. 2010, Witte et al. 2007)

The goals are a reduced degradation rate in order to give the body enough time to deal with the evoluted gas. Moreover, the material has to keep its mechanical integrity over a certain time period to enable fracture healing. (Purnama et al. 2010)

4.3 Overview of the so far tested magnesium alloys

Magnesium-Zinc(-Zirconium):

The element zinc as alloying compound can be considered as harmless, thus being an essential trace element of the human body. Benefits from using zinc as supplement to magnesium alloys are a better yield strength and an inversely proportional gas evolution with an augmented zinc contend. (Persaud-Sharma, McGoron 2012) Cell and Mg-Zn-Zr alloy co-culture showed a higher proliferation rate of the cells after prolonged cultivation compared to other alloys, i.e. a WE-type. (Huan et al. 2010) Cell growth on hydroxyapatite reinforced Mg-Zn-Zr was better than on simple Mg-Zn-Zr, namely a higher cell density was observed. (Ye et al. 2010, Huan et al. 2010)

Magnesium-Calcium:

The alloying element calcium has the big advantage of having a similar density to bone and further to magnesium. It is naturally present and necessary in the human body, thus no toxic effects on cells within a wide concentration range can be expected. Calcium alloying augments the solid solution and acts as a grain refiner. (Xin, Hu & Chu 2011, Li et al. 2008) From a mechanical point of view alloying with calcium over a weight percentage of 5, makes the matter brittle at room temperature, and is therefore inapplicable. Out of this reason just 1, 2 and 3 weight percent of calcium were tested. In vivo the two latter exhibited very fast degradation. The alloy with one percent calcium degraded in vivo within 18 weeks. During this time period and especially in the first month gas evolution was observed. The gas pockets faded with time and did not cause harm to the surrounding tissue. Even a high activity of osteoblasts and osteocytes could be shown. In cell implant (1 wt% of Ca) co-culture viability of fibroblasts increased compared to the control. (Li et al. 2008)

Magnesium-Aluminium:

Aluminium is known as a neurotoxic element that could induce neurological pathologies like Alzheimer disease, senile dementia and dementia. (Li et al. 2008) Furthermore, aluminium related blood-brain-barrier dysfunction had been

observed. As an alloying element it improves corrosion resistance and some mechanical features. (Witte et al. 2005)

Magnesium-Manganese:

The problem that most of the tested magnesium alloys contain eventually toxic or at least not cytocompatible elements is a fact. (Huan et al. 2010) These impurities can accelerate as well as they can slow down corrosion. Manganese, which is a natural trace element in humans, serves if added to magnesium as enhancer of corrosion resistance. This is owing to the reduction of the harming effects of impurities by compounding them.

The usually benign manganese can cause "Manganism" and neurological disorders similar to Parkinson's disease at massive intake. In in vitro cell culture studies a severe cytotoxicity including lower cell viability and a lower proliferation rate was noted. These effects were only seen after adding rather high manganese concentrations. (Xin, Hu & Chu 2011, Persaud-Sharma, McGoron 2012)

Magnesium-Strontium:

The trace element strontium is mainly prevalent in the human bone. It is used in osteoporosis therapy to increase bone mass. Consecutively, the biomedical safety is beyond questions. Used as alloying element it improves, up to 2 weight percent, the yield tensile strength and ultimate tensile strength compared to pure magnesium. Furthermore, it shows in vitro a reduced corrosion rate while activating osteoblast replication and inhibiting osteoblast differentiation. Strontium induces significantly the alkaline phosphatase activity. In vivo higher cortical bone thickness and bone density were demonstrated with magnesium-strontium alloy treatment. (Gu et al. 2012)

Magnesium-rear earth elements:

The somehow misleading name rear earth elements (REE) dates back to ancient times, when these metals were found in form of their oxides, which were formally called earth. (Drynda et al. 2009) The REEs are 15 transition metals in total within group III of the periodic table. They are divided in the three different weight groups: light, medium and heavy. (Hirano, Suzuki 1996) REEs are used in magnesium

alloys with the aim to influence degradation behaviour and gas evolution. Besides, they also enhance corrosion and creep resistance. They are known to have beneficial properties like ameliorated mechanical performance, namely yield stress, tensile strength and elongation. (Li et al. 2008, Drynda et al. 2009, Xin, Hu & Chu 2011)

REEs like yttrium, lanthanum, cerium, praseodymium, gadolinium, neodymium and dysprosium are alloyed with magnesium to fulfil these objectives.

In vitro cell culture testing of commonly used REEs showed neither elevation in apoptosis nor necrosis nor diminished metabolic activity up to a wide concentration range. An exception was investigated concerning some light REEs which showed toxic effects at lower concentration levels. This was demonstrated by reduced viability compared to the control and also compared to the heavy weight REEs. (Feyerabend et al. 2010) However, a dose-dependent augmentation of inflammatory genes like Interleukin (IL) 6, IL 8 and intercellular adhesion molecule (I-CAM) was detected in vitro after treatment with REEs. (Drynda et al. 2009) In vivo studies at which yttrium, cerium and praseodymium were administrated intravenously to rats a severe hepatotoxicity could be shown. (Li et al. 2008)

Also, the use of specific REEs as contrast reagents for magnetic resonance imaging, as anti-carcinogenic and anti-inflammatory reagents against synovitis have been reported. (Feyerabend et al. 2010, Hirano, Suzuki 1996, Hort et al. 2010)

It is problematically that still very little is known about the effects of REEs on the human body. Depending on the way of intake REEs are deposited in different compartments of the body like the teeth, the liver, kidney, soft tissues, spleen, lung or the skeleton. The urinary excretion after injection seems to be dependent on the stability, respectively chelation of the REE. Chelated ones are excreted from the blood rather rapidly, while chlorides are taken up and excreted slowly and difficultly. Especially the clearance from the bone is very slow. The effect on bone growth after treating different animals with varying REEs showed contrastive results. While the growth of mice that had REEs in their drinking water was depressed, they at the same time survived longer than the untreated group. The feeding of rats with REEs showed no significant effects on their growth. (Hirano, Suzuki 1996)

5. Aim of the study

The aim of the study is to evaluate the in vitro cytocompatibility and osteoinductivity of two magnesium based biodegradable materials. In this particular case we focus on the two magnesium alloys ZX50 and WZ21.
The study will be conducted on the human osteoblast cell line MG63 and on expressly harvested epiphyseal cells. Firstly, it is the goal to investigate and show changes of the osteoinductive markers alkaline phosphatase and osteocalcin after incubation for 48 hours with the eluates of the two alloys. Alter of the genes will be inquired by quantitative Real Time Polymerase Chain Reaction (qRT-PCR) and interpreted in terms of their osteoinductive potential. Secondly, the metabolic activity and viability of the cells will be examined with the help of MTT and neutral red test. To examine the parameters, optical density is measured by a spectrometer after transformation and neutral red colour up-take, respectively. The results will also be set in relation with former in vivo studies of the two alloys.

6. Materials and methods

6.1 Materials

The two magnesium alloys used in this study are ZX50 and WZ21, both obtained from the Laboratory of Metal Physics and Technology, Department of Materials, ETH Zurich.

The nominal composition of ZX50 is magnesium balanced, zinc 5 weight percent (wt%), calcium 0,25 wt% and manganese 0,15 wt%, while the composition of WZ21 is magnesium balanced, yttrium 2 wt%, zinc 1 wt%, calcium 0,25 wt% and 0,15 wt% manganese. **(see Table 1)** From former in vivo research it is known, that the alloy ZX50 has a higher corrosion rate with large gas evolution. It is faster dissolving in vitro and in vivo conditions than the WZ21 alloy. The WZ21 pins showed good osteoinductive effects in an in vivo rat model. (Kraus et al. 2012)

Table 1: The nominal composition of the two alloys ZX50 and WZ21 in wt%.

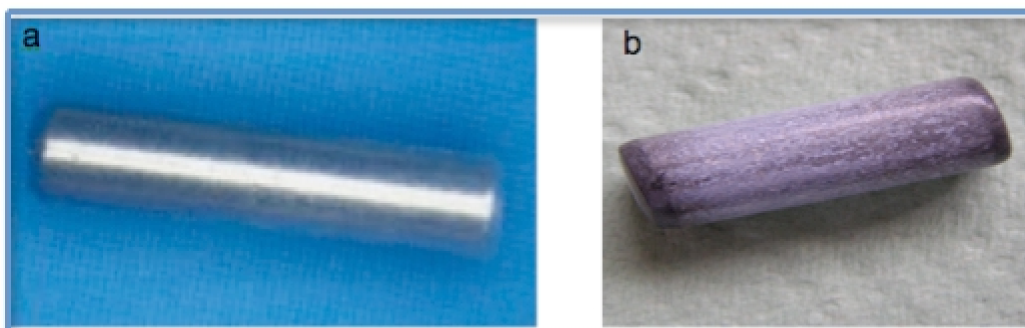
Alloy	Magnesium	Zinc	Calcium	Manganese	Yttrium
ZW50	Balanced	5	0,25	0,15	-
WZ21	Balanced	1	0,25	0,15	2

6.2 Methods

6.2.1 Eluate preparation

The eluates are prepared according to Hänzi et al. (Hanzi et al. 2010)
The cylindrical shaped magnesium alloy-pins **(see Figure 7)** were cut in order to make the surface area 590 mm^2. The pins surfaces were grounded with sandpaper (grit size P 400 with an average particle diameter of 35 μm) to remove corrosion products. In the following, washing in 70 % ethanol for five minutes and three washing steps with sterile filtered (Whatman FP 30/0,45μm, Dassel, Germany) simulated body fluid (SBF) were performed. The washing steps were carried out to dilute the remaining ethanol before incubation. The pins were put in a falcon with 1 ml sterile SBF per 59 mm^2 surface area and shaken on a shaking plate (180 rpm) at a temperature of 37° Celsius for 40 hours. The chosen falcon size allowed the pin to move easily and was constantly dipped into the fluid while shaking. Furthermore, the falcon was sealed with Parafilm (Marienfeld-Superior, Lauda-Königshofen, Germany) to avoid leakage. After 40 hours the pin was removed from the SBF, washed with double distilled water, air-dried and weigh. Pictures were taken to show corrosion of the pins. The eluate was, if not immediately used for experiments, stored at -20° Celsius.

Fig. 7: Magnesium implant pin before (a) and after incubation for 40 hours in sterile SBF at 37° Celsius on a shaking plate (b)



6.2.2 Cells and cell culture

For the experiments the MG63 osteoblasts, obtained from European Collection of cell Cultures (ECACC), and primary human growth plate chondrocytes (hGPC) were used.
The MG63 cells were cultured in Minimum Essential Medium (MEM, Sigma-Aldrich, Vienna, Austria) with supplementation of 10 % fetal bovine serum (FBS, Gibco, Carlsbad, California), 1 % Non Essential Amino Acids (NEAA, Sigma-Aldrich), 1 % Penicillin/Streptomycin, 1 % L-Glutamine (both Gibco) and 0,1 % Amphotericin B (PAA Laboratories, Linz, Austria).
The culture medium used for the primary hGPC was Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12, Gibco) with 5 % FBS, 1 % Penicillin/Streptomycin, 1 % L-Glutamine and 0,1 % Amphotericin B.

Both cells types were cultivated in a humidified chamber at 37° Celsius with 5 % CO_2-content according to cell culture standards. Washing with phosphate buffered saline (PBS) 7,4 pH (Gibco) to remove metabolism products and medium exchange, was performed three times a week.

6.2.3 Cell isolation

The epiphyseal cells were harvested from supernumerary digits of children with polydaktylism. All of them underwent surgery at the Department of Paediatric and Adolescent surgery at the Medical University Hospital of Graz. Immediately after the operation soft tissue surrounding the cartilage was removed from the specimen with a sterile scalpel under the working bench. According to the preoperative x-ray picture, the growth plate was displayed and cut into thin slices. At this, cartilage forming the articular joint was incubated separately from the epiphyseal cartilage. To avoid contamination with osteoblasts the last slice of cartilage before the mineralized bone, was dismissed and not used for cell culture.
After mincing the slices with the scalpel they were digested in 300 Units Collagenase B (Wothington, Biochemical Corp., Lakewood, New York) per 1 ml

culture medium to release chondrocytes from the tissue. The Parafilm sealed falcon was shaken at 150 rpm over night at 37° Celsius. After that the fluid was filtered trough a cell strainer (40 µm nylon, Biosciences Falcon) and centrifuged at 1100 rpm for three minutes. Supernatant was removed and cells resuspended in cell culture medium. After counting the cells with a Casy cell counter TT (Schärfe System, Reutlingen, Germany) they were seeded in a density of 16 000 cells per cm^2 and cultured at standard cell culture conditions at 37° Celsius in a humified atmosphere with 5 % CO_2-tension.
The ethical committee of Graz approved the above-described procedure (EK-No. 20-344 ex 08/09).

6.2.4 Cell treatment and gene expression analysis:

For gene expression analysis cells were seeded in 6-well-plates (Nunclon Vita MultiDish 6, Thermo Scientific) at a density of 4,87 x 10^4 cells per cm^2. 24 hours after seeding, cells were washed with PBS and 5 ml of medium, prepared with 4 % eluate of the total liquid amount, was added. As a control one well treated with pure SBF and one untreated well was used. After incubation for 48 hours at standard conditions, total messenger RNA (mRNA) from samples and controls, were isolated with RNeasy Kit (Quiagen, Hilden, Germany) according to the protocol. To annihilate possible DNA contamination a 30 minute digesting step at 37° Celsius, with 1 Unit DNase (Fermentas, Burlington, Ontario) per gram RNA was performed. The mRNA quality and content was checked with NanoDrop ND-1000 Spectrophotometer (Peqlab, Erlangen, Germany). Samples were stored at -80° Celsius if not used initially. 2 µg of mRNA was transformed in complementary DNA (cDNA) using RevertAid HMinus First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific) according to the instruction. To perform qRT-PCR the Bio-Rad CFX96-Real-Time-System (Bio-Rad, Vienna, Austria) was used. The SYBR Green (Invitrogen, Carlsbad, California) based PCR was pipetted in triplicates in a 96-well-plate with a total reaction volume of 19 µl per probe. The samples were used in a dilution of 1:12,5. 50 ng/µl per sample of cDNA was analysed for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase

(GAPDH), osteocalcin and alkaline phosphatase. All primers were obtained from MWG, Ebersberg, Germany. The primer sequences are listed in table 2.

Table 2: Human primer pairs used for qRT-PCR.

Gene	Sequence forward, reverse
GAPDH	5'-AAGGTCGGAGTCAACGG-3', 5'-ACCAGAGTTAAAAGCAGCCCT-3'
Osteocalcin	5'-GGCGCTACCTGTATCAATGG-3', 5'-TCAGCCAACTCGTCACAGTC-3'
Alkaline phosphatase	5'-CACCAACGTGGCTAAGAATG-3', 5'-ATCTCCAGCCAGCCTGGTCTCCTC-3'

6.2.5 MTT and neutral red test:

To estimate the cytocompatibility, namely the viability and the metabolic activity of the cells after treatment with the two alloy eluates, MTT and neutral red testing were performed. The MTT test is based on the conversion of (3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyltetrezoliumbromid and represents the metabolic activity of the cells. The neutral red is physiologically taken up into the lysosomes of the cells and gives insight into their viability.
For both experiments cells were seeded in a 48-well-plate (Nunc Multidishes Nunclon, Thermo Scientific) at a density of 60 000 cells per well. The culture surface of each well is 1,1 cm^2. To allow settlement of the cells, they were treated 24 hours after seeding with the ZX50 and the WZ21 eluates. This was carried out at concentration ranges between 0,4 and 40 percent of the total liquid amount. To evaluate the influence of the conveying fluid, the cells were treated with the same amounts of pure SBF. Untreated cells served as the control. For the MTT and the neutral red test the probes were measured after 48 hours subsequent to the treatment. The measurement of the optical density was carried out at a wavelength of λ = 550 nm on a SPECTROstar Omega luminometer (BMG Labtech, Germany).

6.2.6 Statistical analyses

The formula: threshold cycle (Ct) = Ct of target gene − Ct of GAPDH was employed to calculate the difference in expression between the reference and the target gene in qRT-PCR. A negative value represents a reduced, a positive value an augmented expression of the gene compared to the normal cellular activity.

The SPSS® 19.0 software (SPSS Inc., Chicago, Illinois) was utilized for statistical analyses. To work out statistically significant differences, which were determined at a p-value < 0,05 between the treated and control groups, the Wilcoxon signed-rank test was performed.

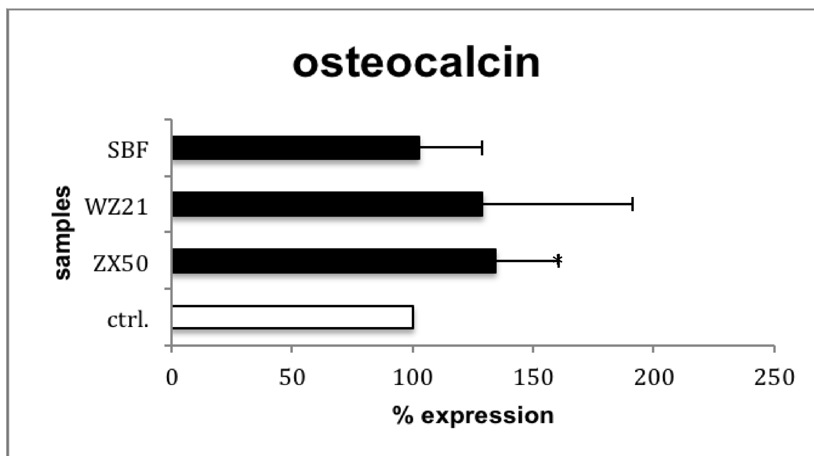
7. Results

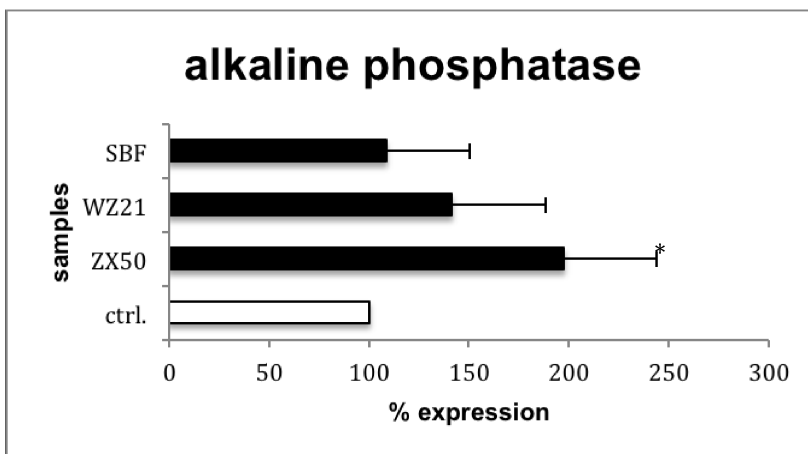
7.1 Gene expression analyses

MG63:

The gene expression analysis for the MG63 osteoblasts after 48 hours of incubation with the ZX50 eluate showed significantly elevated alkaline phosphatase and osteocalcin levels compared to the control. MG63 cells treated with the WZ21 eluate for the same time period showed altered but not significantly higher levels of alkaline phosphatase or osteocalcin. **(see Figure 8)**

Fig. 8: Changes in the mRNA expression of alkaline phosphatase and osteocalcin in MG63 osteoblasts after 48 hours of incubation. Significance was assumed when p was less than 0,05 (labelled with an asterisk).

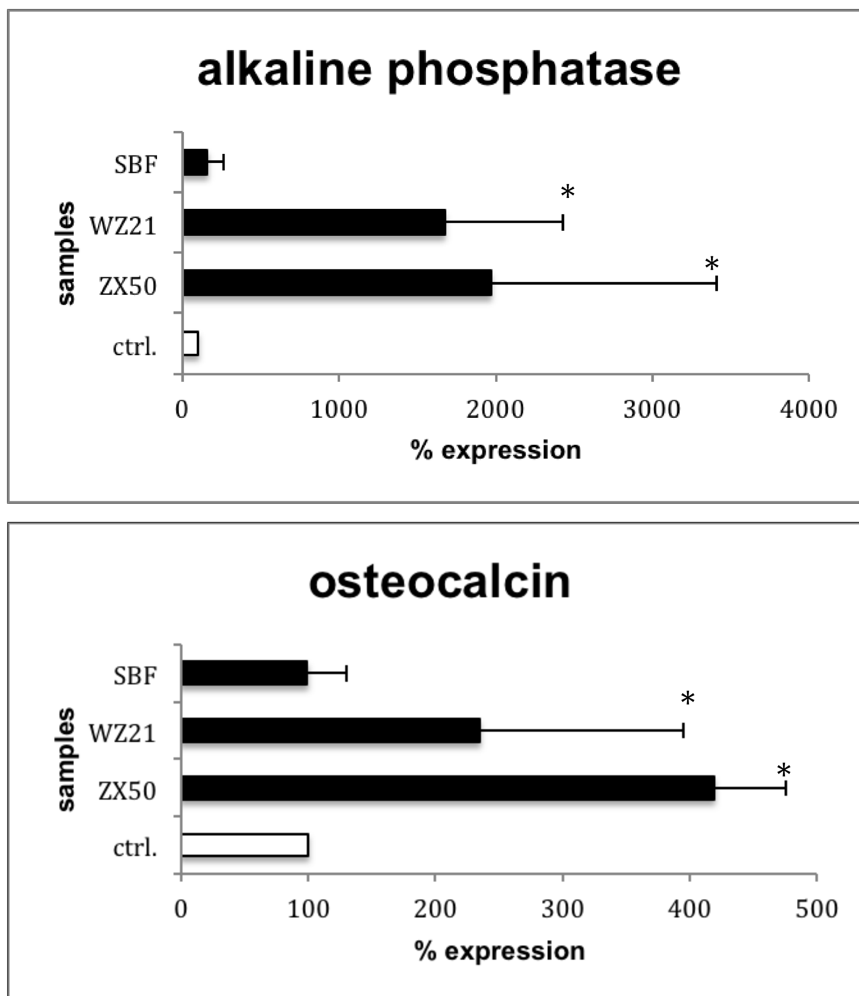




hGPC:

The mRNA levels of the osteogenic markers alkaline phosphatase and osteocalcin were significantly augmented for both, ZX50 and WZ21 eluates, after 48 hours of incubation. The gene expression of ALP and osteocalcin was compared to the untreated and the SBF treated control. **(see Figure 9)**

Fig. 9: Changes in the mRNA expression of alkaline phosphatase and osteocalcin in hGPC after 48 hours of incubation. Significance was assumed when p was less than 0,05 (labelled with an asterisk).

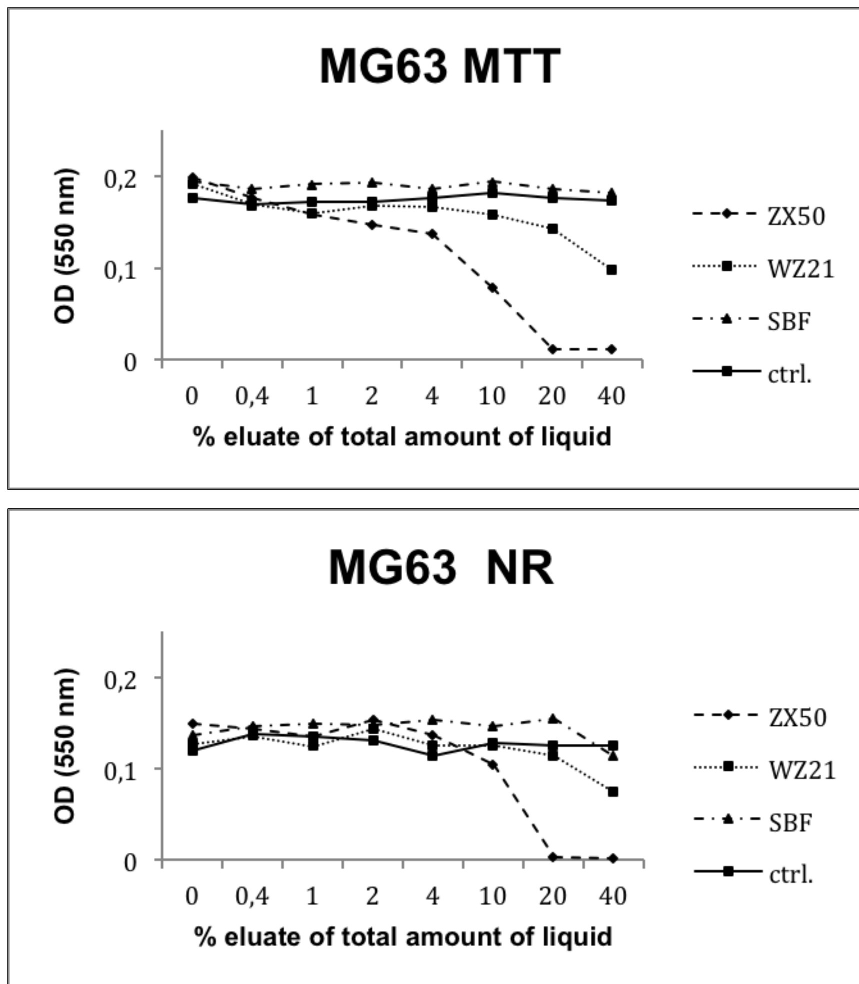


7.2 MTT and neutral red test

MG63:

In the case of MTT and NR testing a lower optical density has to be equated with a decrease in metabolic activity and viability, respectively. The MG63 osteoblasts came out to be very resistant against relatively high amounts of the eluates. With the addition of the ZX50 eluate the metabolic activity as well as the viability did not drop until a concentration of 20 percent of the total liquid amount was reached ($p = 0{,}000$ and $p = 0{,}006$, respectively). After treating the cells with the WZ21 eluate neither a significant decrease in metabolic activity nor in viability was measured. This was the case for the whole tested concentration range. Equally, the treatment with SBF had no significant influence on the viability or metabolic activity of the cells. **(see Figure 10)**

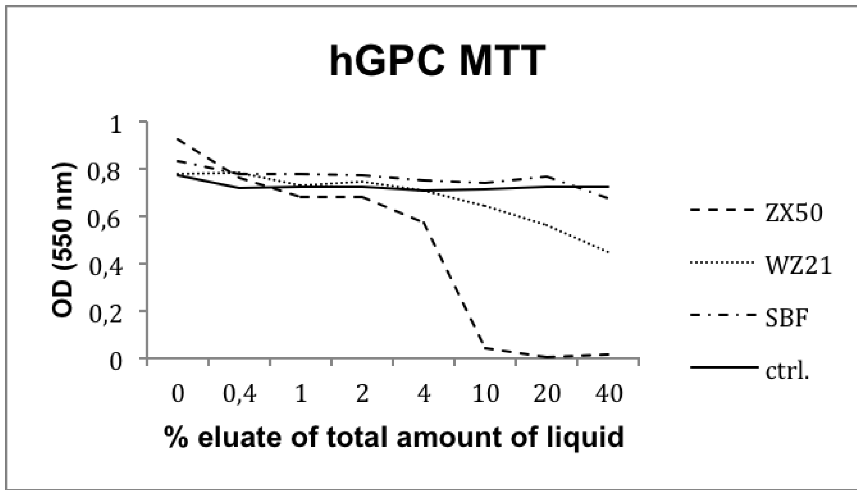
Fig. 10: Metabolic activity and viability of MG63 osteoblasts tested by MTT conversion and lysosomal neutral red take-up.

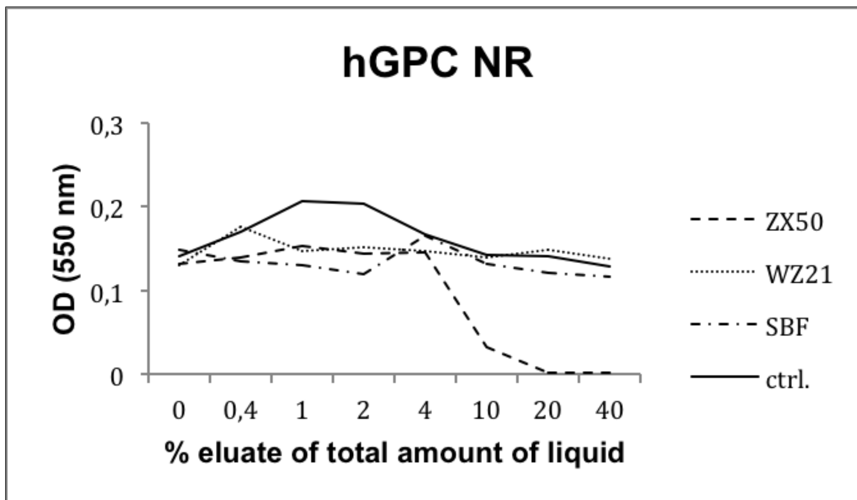


hGPC:

The hGPC showed a decreased viability in the NR test and also a decreased metabolic activity in the MTT test after treatment with the ZX50 eluate as soon as the concentration exceeded 10 percent of the total amount of liquid (both p = 0,000). Both, the WZ21 and the SBF treated cells showed no measurable effects in terms of metabolic activity or viability. **(see Figure 11)**

Fig. 11: Metabolic activity and viability of hGPC tested by MTT conversion and by lysosomal neutral red take-up.





8. Discussion:

The results of the study showed a good in vitro cytocompatibility for the two magnesium alloys ZX50 and WZ21. Particularly the slower degrading WZ21 was well tolerated by both cell types in terms of metabolic activity and cell viability. The cells treated with the ZX50 eluates showed a higher sensitivity towards this implant material. This could be because of the higher and more remarkable increase in pH when dissolving the faster degrading alloy ZX50. The relatively high pH levels are due to the reinforced release of metallic ions from the latter mentioned material. The released ions cause the alkalization of the fluid. (Huan et al. 2010) It is known that pH levels over approximately 7,6 can alter biosynthetic activity of osteoblasts. Alkaline phosphatase activity, lactate production and DNA content of the cells can also be influenced by an elevated pH level. (Kaysinger, Ramp 1998) The slighter increase of the pH level could be an explanation for the better cytocompatibility of the WZ21 alloy.

Another research group who did cytocompatibility studies on rat derived primary osteoblast cells also detected a better cytocompatibility of their slower degrading alloy. In this special case a Mg-Zn-Zr alloy was compared to a hydroxyapatite reinforced composite. In contrast to this study they used a direct investigating method, in detail a cell-implant-co-culture, and observed the following in a timeframe of one week. On the surface of the slower degrading composite, the HA reinforced one, a higher cell density and a higher cell activity was found. Additionally, after 5 days of incubation almost no viable cells could have been detected on the faster degrading alloy. The cause for this observation was hypothesised to be the faster degradation and the strong pH level increase. (Ye et al. 2010) If applied to this study the longer co-cultivation and the higher concentration in the MTT and NR test could be set alike. Meaning the rapidly corroding material causes higher pH level augmentations, therefore lower viability and activity of the cells.

A research group whose focus laid on the application of biodegradable magnesium alloys as well as on vascular stents investigated the cytocompatibility of the WZ21 amongst other alloys. Human umbilical vein endothelial cells

(HUVEC) were used in that case for the MTT and NR tests. Their results showed a decrease of metabolic activity in the MTT test as soon as the WZ21 eluate content exceeded 4 percent of the total amount of liquid. The viability of the treated cells dropped at a negligibly higher concentration. (Hanzi et al. 2010) In our study neither cell viability nor metabolic activity of MG63 or hGPC were greatly influenced throughout a concentration range of 0,4 to 10 respectively 15 percent of the total liquid amount. Although, we used the same method for the eluate preparation, the incubation time diverges from the other study. In this investigation the cells were incubated for 48 hours while in the other case the incubation lasted for seven days. Two possible explanations for the contrastive results are the following. Firstly, the HUVEC cells were treated for a longer time period or secondly the HUVEC are more sensitive towards this kind of treatment. Moreover, in our study we could not find the, from Hänzi et al. announced connection between the dilution of the culture medium with the eluate or the SBF and a reduced viability and metabolic activity of the treated cells. In their case an increase of metabolic activity in the control group, namely the untreated cells was found. This would mean that the released metallic ions per se do not harm the cells. (Hanzi et al. 2010) A probable explanation why we did not detect this phenomenon is that the time of our treatment was to short.

In terms of the frequently described increase of bone mass and mineralization of the callus in the immediate vicinity of a magnesium-alloy implant in vivo (Witte et al. 2005, Kraus et al. 2012, Li et al. 2008, Yamasaki et al. 2003) this study could demonstrate that these observations have a correlation with mRNA levels of osteogenic markers in vitro. Especially the ZX50 alloys' eluate induced a significant increase of the two osteogenic markers alkaline phosphatase and osteocalcin in both the MG63 osteoblast cell line and the human growth plate chondrocytes. The WZ21 alloys' eluate effected an augmentation of the osteogenic markers too, although it was just significant for the hGPC and not for the MG63 cells. This augmentation is appreciable especially for the osteoblasts. In terms of the hGPC it could indicate an unfavourable starting point for an untimely ossification of the growth plate. A plausible and obvious explanation why ZX50 induces a stronger increase of osteogenic markers is that, due to the faster

degradation, more magnesium ions are released into the fluid. In other studies it could be demonstrated that not just an increase of osteogenic markers in the cells but also a stronger in vivo proliferation of the osteoblasts, a higher activity of osteocytes and a speedup of callus mineralization could be induced by a moderate magnesium release. (Li et al. 2008, Huan et al. 2010, Feyerabend et al. 2006) It was also reported that a moderately augmented magnesium level has a stimulatory effect on the proliferation and redifferentiation of primary chondrocytes. This effect was outlined to inverse when a certain magnesium concentration was exceeded. (Feyerabend et al. 2006) This would mean that the magnesium release of the two alloys ZX50 and WZ21 is not too extensive.
An interesting though controversial study on a MG63 cell line, whose main goal was to investigate the toxicity of different but frequently orthopedically used metals, did not report a higher proliferation rate of MG63 osteoblasts with the addition of magnesium. The proliferation was set in relation to that of untreated cells. Metallic ions that induced an augmented proliferation of the MG63 cells in that study were chromium, cobalt, molybdenum and tantalum. (Hallab et al. 2002)

This in vitro study is of particular importance as due to the cell experiments numerous experiments on animals were avoided. Still it has to be clear that in vitro test can only be supplements but never replacement of in vivo studies. Another favourable detail of this inquiry was, that human cells, instead of the often-used mouse chondrocyte cell line, were utilized for the cell culture experiments. The limiting factor with the MG63 cell line is, the same as with every other cell line, that cell lines are genetically modified. This modification often makes the cells more robust against noxious or experimental influences. It is also within the bounds of possibility that a cell line's reaction to the experimental exposures differs extensively from a primary cell because their typical in vivo characteristics could not be prevented. In return the hGPC are primary cells, that is why their reactions should be closest to the ones of in vivo conditions. Research on primary cells is considered as something in between cell line and in vivo experiments. (Schmitz 2009) Out of this reason the expressly harvested human growth plate chondrocytes along with the MG63 cells should be a good model to investigate our purpose. The weak point of the hGPC is that they are collected from

supernumerary digits. For this reason it cannot be assured that these cells do not diverge genetically from "normal" hGPC.

To summarise the findings: The osteoblast cell line as well as the primary growth plate chondrocytes seemed to be pushed towards mineralization by the elevated magnesium content in their surrounding. This finding can be considered positively for the MG63 osteoblasts but not necessarily for the hGPC. Moreover, the faster degrading ZX50 alloy may induce a relatively strong pH level increase. This high pH level restricts the cells in terms of viability and metabolic activity. Therefore, the WZ21 alloy seems, despite the Yttrium compounding, the more relevant material for further investigations.

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