PhD thesis

Enhance bone regeneration and implant fixation by novel substitute with combined angiogenic and osteogenic factors in animal models

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Contents

List of abbreviations

ACL: Anterior cruciate ligament

Ang: Angiopoietins

ANOVA: Analysis of variance

ARCO: Association research circulation osseous

AVN: Avascular necrosis

BMP: Bone morphogenetic protein

BMU: Bone remodeling unit

BSA: Bovine serum albumin

BV/TV: Bone volume / tissue volume

CDHA: Calcium-deficient hydroxyapatite

CFU: Colony forming unit

CNS: Central nervous system

CSD: Critical size defect

DMSO: Dimethyl sulfoxide

EC: Endothelial cells

EDTA: Ethylenediaminetetraacetic acid

FBS: Fetal bovine serum

FDA: Food and drug administration

FGF: Fibroblast growth factor

HA: Hydroxyapatite

IL: Interleukin

I.m: Intramuscular

IP: Intraperitoneal

IRA: Innovative Research of America

kDa: Kilodalton

LPS: Lipopolysaccharide

MEMA: Minimum essential medium alpha

MMA: Methyl methacrylate

MPS: Methylprednisolone

MSC: Mesenchymal stem cells

N: Newton

NSAID: Non-Steroidal Anti-inflammatory Drug

OB: Osteoblast

OC: Osteoclast

OVX: ovariectomy

PDGF: Platelet derived growth factor

PDLLA: Poly(D,L-Lactide

PSG: Penicillin streptomycin glutamate

RCF: Relative centrifugal force

ROI: Region of interest

RRR: 3 R's Replacement, reduction and refinement

rVEGF165: Recombinant human VEGF165

S.c.; Subcutaneous

SCID: Severe combined immunodeficient

SD: Standard deviation

SPF: Special care pathogen free

SVF: Stromal vascular fraction

TGF-B: Transforming growth factor beta

THA: Total hip arthroplasty

TNFα: Tumor necrosis factor-α

TNF- β: Transforming growth factor-beta

VEGF: Vascular endothelial growth factor

VOI: Volume of interest

Preface

This thesis consists of 3 papers. Additionally, it briefly mentions 2 future studies in their current states and for future direction of this work.

List of papers

Study 1

Dreyer CH, Kjaergaard K, Ditzel N, Jørgensen NR, Overgaard S, Ding M. Optimizing combination of vascular endothelial growth factor and mesenchymal stem cells on ectopic bone formation in SCID mice. J Biomed Mater Res Part A. 2017;105(12):3326–32.

Study 2

Dreyer CH, Jørgensen NR, Overgaard S, Qin L, Ding M. Combination of vascular endothelial growth factor and mesenchymal stem cells for bone formation in a sheep model (In manuscript)

Study 3

Dreyer CH, Kjaergaard K, Ding M, Qin L. The use of vascular endothelial growth factor for in vivo bone formation: A systematic review (In manuscript)

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Dansk resumé

Knogledannelse har fået en forøget interesse i ortopædkirurgisk forskning grundet prædiktionen om en stigning i gennemsnitalderen i befolkningen. Dette betyder der bliver flere patienter med svagere knogler og derved større risiko for knoglebrud med sværere heling. Dette gør vi klinisk fremadrettet har brug for en holdbar og effektiv behandling til disse patienter, for at kunne sikre sufficient og hurtigere knogleheling, færre komplikationer og nedsat behov for re-operationer.
Den nuværende behandling for større knogledefekter er at bruge allograft. Dette er overskydende knogle som er indhentet fra hoftealloplastikker eller kadavere som er doneret til formålet. Men denne metode er forbundet med risikoen for at overføre sygdomme med knoglen samt risikoen for at der opstår immunreaktioner, som man ser ved organdonation. Derudover, kan knoglebankerne som opbevarer allograft ikke følge med den stigende efterspørgsel.
Et alternativ til denne behandling kan være at bruge biomaterialer som denne afhandling fokuserer på. Disse har fordelen af ikke at kunne overføre nogle sygdomme og kan produceres til behovet. Teorien bag designet var at stimulere de kritisk vigtige faktorer i knogledannelsesprocessen med samtidig indflydelse af kardannende vækstfaktorer, dog kun med fokus på knogle- og ikke kardannelse. Dette var testet ved den naturlige udvikling i dyrestudier hvor vi først afprøver og optimerer metoderne i smådyr, for at videreudvikle til stordyr. Vi startede med at teste denne knogle- og kardannende stimulering med mesenkymale stamceller (MSC) og den karstimulererende vækstfaktor (VEGF) i et ektopisk design på mus, hvor fokus var hvilke tidspunkter der var bedst at stimulere MSC med VEGF for den største indvirkning på ny knogledannelse sammenlignet med kun at bruge MSC (studie 1). Adskillige pilotstudier indikerede mængden der skulle bruges af MSC og VEGF samt evalueringstid og racen af musene. Dette studie viste at den optimale stimulering med VEGF kombineret med MSC var indenfor de første 1-14 dage og 1-21 dage efter operation, sammenlignet med MSC alene $p<0.01$.
Studie 2 brugte samme kombination af MSC og VEGF, men denne gang i en femoral gap model i får. Får har vist sig at være en god model for ortopædisk knogleforskning da både vægten, knogleindholdet og metabolismen er meget lig det humane. Et pilotstudie gav indikationer for mængden af MSC og VEGF i dette design. I det primære studie testede vi forskellige doser af VEGF kombineret med MSC sammenlignet med allograft. I denne model fandt vi ingen signifikant forskel imellem nogle af grupperne $p<0.05$.
Studie 3 begyndte overvejelserne omkring videreførelsen af disse metoder i et klinisk sammenligneligt design. Dette med overvejelsen omkring om VEGF alene kunne være nok til at

skabe sufficient knogledannelse. Derfor fungerer dette studie som et springbræt til fremtidige studier. Efter en systematisk litteratursøgning, kunne dette dog ikke bekræftes fra tidligere studier.

Konklusionen fra disse studier viser vigtigheden af sufficient karforsyning i et område specielt i forbindelse med knogleheling. Derudover, at hvis man bruger mesenkymale stamceller og kar stimulerende vækstfaktor (VEGF) kan man hele kritiske defekter på lige fod med allograft. Dette stiller spørgsmålet om VEGFs rolle i knogledannelse og om man med standardiserede metoder kan applicere det i klinikken for at forbedre knoglehelingen både i normal, osteoporotiske og avaskulær nekrose patienter. Denne teknik kunne ved yderligere forskning lede til human applikation i den nærmeste fremtid samt optimeret knogledannelse, indvækst og stabilitet i generel osteosyntese.

Summary

Bone regeneration is attracting an increasing level of interest in the field of basic orthopaedic research due to the prediction of increasing fracture incidence resulting from a growing elderly population coupled with the need for a sustainable and unlimited method to ensure optimal bone healing. The clinical need for such an application is increasing, with the aim of ensuring fewer complications and shorter immobilisation periods for bone fracture patients.

The current method of bone regeneration used in larger bone defects is an allograft harvested from the bone of a donor after the insertion of arthroplasties, or from cadavers. However, this method is associated with the risk of disease transmission and immunogenicity. Furthermore, the bone bank storage of allografts cannot keep up with the clinical demand.

In the present work, biomaterials were used in an attempt to develop an alternative to these complications. The theory behind this design was to enhance critical factors in the bone remodelling process with an influence of angiogenic stimulation, but with a focus on bone growth only. This theory was tested via the natural development of animal studies from rodent to large animal models.

We started by testing the theory in a severe combined immunodeficient (SCID) mouse ectopic bone model in Study 1 using mesenchymal stem cells (MSC) in combination with vascular endothelial growth factor (VEGF) at different time points. We evaluated if MSC combined VEGF was better at enhancing bone than MSC alone. Several pilot studies were performed to give an indication of the optimal dosages of MSC and VEGF in SCID mice and to evaluate a suitable time for the delivery of VEGF in this design. The study demonstrated that the optimal time of VEGF administration was within the first 14 and/or 21 days after operation in combination with MSC when compared to MSC alone ($p<0.01$).

Study 2 then attempted the same combination of MSC and VEGF, except in a femoral implant gap model in sheep. Sheep have proven to be a good model in the field of orthopaedic research due to their body weight, bone content and bone metabolism. A pilot study provided an indication of the amount of MSCs that would be preferable to administer with VEGF. In the primary study, we tested different doses of VEGF combined with MSC and compared them to the allograft method. Notably, we found no difference in bone formation in our gap between both groups ($p<0.05$).

Study 3 investigated further research and development (R&D) purposes and attempted to determine whether current progress within this field suggests that the stimulation of VEGF alone was sufficient for optimal bone formation. While this study clearly serves as a stepping stone for future project designs, a systematic literature search found no conclusive results regarding whether VEGF alone is sufficient for promoting bone growth.

In conclusion, the importance of blood vessels in the environment is an essential factor in enhancing bone regeneration. Furthermore, stimulating both angiogenic and osteogenic properties in bone healing using the right methods can heal a critical size defect (CSD) with the same amount and quality as an allograft. This provides another indication for the use of VEGF in the field of bone regeneration for patients with osteoporosis and avascular necrosis, with further investigations being required to expand on this topic. By further studying with the focus of human applications in the near future, this technique could facilitate optimised bone ingrowth and the stabilisation of general osteosynthesis.

Background

Clinical problem

The issues of fracture healing and bone defects have always been a focus within orthopaedic surgery. Ideally, defect healing should be faster, cheaper and generate better results. In accordance with an increasingly elderly population (in the Danish population) and the increased costs of fractures (1) and complication/revision surgeries will rise, resulting in both a health care and a socioeconomic focus in this area of research (2).
This context potentially implies that the bone structure of patients requiring surgery will be worse due to increased age with a higher incidence of osteoporosis. This factor should be considered since osteoporosis will reduce bone porosity, thus negatively affecting the ingrowth of implants (3). The immobilisation period for patients is also critical to their functional rehabilitation, which highlights the greater relevance of early bone ingrowth, as it has been documented as a predictor for implant failure in both stems and arthroplasties (4,5). This suggests that we should drive our focus towards definitions of osseointegration such as biocompatible and load-resistant materials the general strength. Furthermore, we must also consider the status of patient bone tissue, that implant surfaces should be attractive for osteoblasts and that treatments should be as minimally invasive as possible (6).

In the longer term (beyond the next 10 years), this focus on bone formation requires technological advances that maximise cell retention, viability, vascular network formation, osteogenic differentiation capacity and tissue assemble properties to achieve maximum impact on clinical practice (7).

Animal models

When performing *in vivo* studies, it is always important to consider the 3 Rs: replacement, reduction and refinement. This is why it remains essential to have an adequate study design that is as minimally invasive as possible while also considering the correct research model. We began our research by testing our theory in a small animal model via subcutaneous placement with the least inconvenience to the animal. Furthermore, each group consisted of additional pouches to minimise the number of animals used.

When we showed an enhanced effect in the first study, we took moved on to a large animal model. Large animal models can include goat, dog, pig, sheep and even emu, depending on the purpose of the research. Notably, sheep is one of the most used models within the field of orthopaedics due to their similar body weight and long bones, which are comparable to those of humans (8). This allows the use of a realistic implant size during design and surgery. However, it should be noted that sheep have previously been evaluated to have a large amount of trabecular bone in the distal femur, which results in more bone ingrowth (9). Yet, in all sheep studies, allograft serves as reliable control in the same type of bone structure.

General bone healing

Bones are classified as a living organ. The replacement of old bone matrix is a continuous process that maintains bone function and continuity throughout adulthood. This bone adaptation cycle is described through the bone remodelling unit (BMU). This cycle consists of five phases: initiation, bone resorption, active reversal, bone formation, and termination (10). Depending on comorbidities, medical inducement or age, these phases can have different outcomes in terms of bone loss. This cooperation is described as balanced and/or coupled bone formation. If the balance between bone resorption and bone formation are negative, bone loss will occur. This will result in a highly decreased to non-existent bone formation phase, by inhibiting MSC combined with the decrease of amount and function of OB cells (11,12). For example, this can be seen in postmenopausal women or in patients induced with frequent glucocorticoids, depending on administration and dosage (13–15).

Bone healing

Bone healing is a cooperation between many factors, including the integration of different cells and growth factors activated by signalling that is affected by the local environment (16). Bone fractures are categorised by primary/intramembranous healing or secondary/endochondral healing. Primary healing occurs when fractures are not dislocated and the fracture line is barely visible. This is typical in the mandible, clavicula or sternum (17). Secondary healing is the most common and is characterised by the formation of a haematoma at the fracture site (18). Fracture healing consists of different phases that are correlated to each other: inflammation (~24 hours to 7 days), soft callus formation (~days 7–9, peaking at day 14 (in animal models)), cartilage turnover and bone remodelling (begins at week 3–4) (18).

An injury initiates the acute inflammatory response, which causes a haematoma to coagulate and serves as a template in the callus formation phase. Then begins the highly regulated secretion of proinflammatory molecules such as interleukin-1 (IL-1), IL-6, IL-11, IL-18 and tumour necrosis factor-α (TNFα). These factors recruit inflammatory factors and promote angiogenesis while helping with the differentiation of MSCs into an osteogenic lineage (18).

The opposite of this—the inflammatory phase—is a very important factor in the success and timeframe of bone healing (19). This phase can be inhibited by glucocorticoids and Non-Steroidal Anti-inflammatory drug (NSAID) treatments (19,20) and should thus be used with caution when working with both fracture patients and animal models.

Graft material

In bone research, various types of graft materials are used. For example, autograft is harvested and inserted into the same patient. Autograft bone is defined as a "living" material bearing osteogenic, osteoinductive and osteoconductive properties (21). Alternatively, allogenous bone is gathered from non-living bone, which is convenient and without side effects. However, this graft material has mainly osteoconductive effect (22) and carries the potential risk of bacterial infection, disease transmission, autoimmune host response and graft host rejection causing non-unions. While these side effects are more severe, they are extremely rare (23,24).

Notably, harvesting autograft bone is an additional invasive procedure that comes with side effects and the amount extracted is often insufficient. Autograft collected from the iliac crest bone graft (AICBG) can be associated with morbidities such as donor site pain, blood loss, risk of infection and nerve injuries (25). Moreover, the failure rate for this procedure has been shown to be 50%. This rate is caused by the different types of harvesting, handling, implantation methods used. Furthermore, differences between bone vitality and patient conditions also contribute to this high failure rate (26). Due to this failure rate and possible side effects, allogenous bone material is often used as an alternative graft material.

Another option that could diminish the need for harvesting live bone graft is a possible substitute that could be used to fill defects (21,27). The substitute approach has been widely investigated by using combinations of growth factors in animal and clinical models (28) as well as the use of stem

cells from different tissues (29). However, to date, no substitute has been able to replace all of the procedures using autograft and allograft in the clinic.

Substitute

In Studies 1 and 2, we used pure hydroxyapatite (HA), which is a crystalline complex of phosphate and calcium and inorganic material that makes up ~70% of calcified bone. The granule shape was utilised in studies using implant bone blocks in mice as well as gaps in sheep. Pure HA was used due to its high mechanical strength and good biocompatibility in clinical use (30). Clinical studies in humans have also shown good short- and long-term effects of HA used in bone defects. This makes the use of this substitute reliable for future studies (31) and the development of human trials. Granule sizes ranging from 1–2.5mm were primarily used due to our femoral gap implant design, where the concentric gap around the implants had to be filled with a substitute. The granule further provides the possibility of reusing the same type of substitute in a ratio to dosage for different locations and treatment purposes, which strengthens the translational aspect of this research; therefore, standardised blocks was not used.

The total absorption period for pure HA is suggested to be over than 2 years, depending on the amount. The existing literature also suggests the period of resorption to exceed 2–3 years (32). This implies that all HA added in our designs will not have a reduction in the amount of substitute due to observations being limited to 8 and 12 weeks.

Reagents

Mesenchymal stem cells (MSCs)

MSC can derive and be differentiated from all kinds of tissue from bone marrow stem cells (BMSC), adipose-derived stem cells (ADSC), muscle-derived stem cells (MDSC) or embryonic stem cells (ESC) (33). These are multipotential cells with the ability to differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes and myoblasts, depending on the stimulation (34). The objective of the design in this thesis was to stimulate the osteoinductive lineage and, more importantly, trigger this differentiation using the stimulation of VEGF (35,36).

In microbiology, the term mesenchymal stem cell (MSC) is under much debate. Notably, the non-skeletal potential of single MSCs has not been formally proven *in vivo*, with this point remaining controversial (37). Furthermore, some journals demand the use of the minimal criteria set by the

International Society of Cellular Therapy (ISCT), which requires that MSCs be positive for CD70, CD90 and CD105 while being negative for CD34 (38). Some criteria even evaluate this in three stages, where the cells must furthermore lack the expression of CD45, CD73, CD14, CD11b, CD79α or CD19 and HKA class III (39). The most commonly used method of defining an MSC is to check if it is plastic-adherent, which is termed a multipotent mesenchymal stem cells by the ISCT but is still called an MSC. Citation: *"To address this inconsistency between nomenclature and biologic properties, and to clarify the terminology, we suggest that the fibroblast-like plastic-adherent cells, regardless of the tissue from which they are isolated, be termed multipotent mesenchymal stromal cells, while the term mesenchymal stem cells are used only for cells that meet specified stem cell criteria. The widely recognised acronym, MSC, may be used for both cell populations, as is the current practice; thus, investigators must clearly define the more scientifically correct designation in their reports"* (40). As such, the term MSC will be used as the term for multipotent mesenchymal stromal cells in this thesis.

Vascular endothelial growth factor (VEGF)

The vascular endothelial growth factor (VEGF) protein is of particular interest in bone regeneration due to its primary ability to induce neovascularisation (10,15,22), chemokine affection for endothelial cells (ECs) (35) and the differentiation of osteogenic properties (35,36).
Notably, VEGF is considered the main regulator of vascular formation (44). Different cell types, including pericytes, osteoblasts, and smooth muscle cells, secrete VEGF within or around the bloodstream (45). The VEGF family consists of VEGFA,B,C,D and PIGF1,2 (35). We have chosen to use VEGFA due to its increased potency towards angiogenesis (24). Increased blood perfusion around a fracture healing site will enable osteoprogenitors, oxygen, minerals and nutrients from the bloodstream to enter the area of regeneration (47,48), while too many can stimulate malformed and non-functional vessels (41) and high doses can have a toxic effect (49). The VEGF protein also carries the secondary ability to differentiate MSCs into an osteogenic lineage (36,50,51). These effects contribute to achieving a more controlled use of both the stimulating effect of VEGF as well as the regenerative effects of MSCs.

VEGF has two primary receptors: VEGFR1 and VEGFR2 (vascular endothelial growth factor receptors 1 & 2, respectively). VEGFR2 is also known as tyrosine kinase receptor flt-1(52). VEGFR expression from osteoblastic cells (OB) can differ within animal species, humans and depending on cell isolation (53). For example, studies have shown that murine mesenchymal

progenitors and osteoblasts (OB) do not change in response to VEGFR, and mice with the selected deletion of VEGFR in OB cells exhibit reduced bone density two weeks after birth. This could suggest that VEGFR has a positive effect on general skeletal development (54). These findings suggest that both VEGFR1 and VEGFR2 are positive regulators of skeletal development (55). VEGF receptors are not only established in the endothelial cells, which allows them to influence different types of tissue growth. They are located in neurons (56), muscle cells (57) and in osteoblasts (58). This facilitates direct and indirect effects on various tissues that should be considered in the stimulation process.

For optimal bone formation in a fracture model, some of the most important parameters include mechanical stability and adequate blood supply (18). Notably, VEGF plays a role in many of the bone-forming phases. In the inflammatory phase, VEGF is concentrated in the haematoma, while hypoxia will increase the expression of VEGF and neutrophils will stimulate vessels and remove microbial pathogens (59). During cartilage turnover, VEGF has indicated the ability to differentiate the skeletal progenitor cells to enhance the osteogenic (instead of the chondrogenic) lineage. This is consistent with the conclusion that VEGF stimulates periosteal progenitor cells to OB cells (59,60). When the osteoblast is maturing, the secretion of VEGF will rise, which will stimulate more OB cells and result in a continued positive cascade. Furthermore, in endochondral ossification, hypertrophic chondrocytes induce further VEGF expression (61,62).

However, while VEGF can have a positive effect on both the angiogenesis and the migration of endothelial cells, it can also cause oedema by increasing permeability and even the leakage of vessels (63). The effect and dose for this outcome can vary between different tissues (64), and various studies have attempted to regulate the expression of oedema and angiogenesis in downstream signalling with Src (63,65). This could indicate that the therapeutic window of VEGF in tissue regulations is narrow. If the correlation between angiogenesis and permeability should result in increased blood supply for the targeted area, it is very dosage-dependent. This theory could be assumed as the reason for very divergent results in the use of VEGF for bone regeneration.

Recombinant human VEGF165 is a type of VEGFA and has a half-life from 4 (66) to 24 hours (67). This makes it difficult to induce operatively for a long-term effect without reaching the toxicity threshold. As such, the release of this product is a major obstacle in working with VEGF, which highlights the need for some type of release modification—whether it is a method for release or a combination with carrier proteins.

In this thesis, VEGFA will be combined with bovine serum albumin (BSA) at a ratio of 1:50 to prolong the release of the protein. This carrier protein is frequently used as a stabiliser for other solubilised proteins and binds water, salts, vitamins, fatty acids, hormones and carries these bound components between tissues and cells, thereby stabilising the binding of the protein (68,69). The release of the VEGF protein will be performed by validated pellets and coating.

The motivation for this project

When conducting basic research, the primary goal and perspective should always be enhancing treatments or outcomes for patients. Therefore, the question of which challenges are relevant to solve, the understanding of the basic mechanisms and the impact of the research on clinical practice must always be considered.

In some areas of research, the gap between what clinicians prioritise and what full-time researchers focus on has been too separated. While this context produces a lot of quality research, much of it cannot be used in clinical practice due to practical matters such as cost or logistics (70). In this thesis, we attempted to focus on a broad approach to the clinical problems of bone defects, such as implant loosening, delayed healing and an alternative to allograft limited stocks. The design was created to enhance our methods at each step and thereby make preliminary conclusions to be used in the next design.

However, research is a delicate process; therefore, considering current trends, what people are interested in knowing, which materials are suitable to use, what is logistically possible to use in a clinical setting and what has an impact on the research community remains critically important. While differentiated stem cells are difficult to use on a daily basis in the clinical setting due to the timing perspective, they are well known and highly popular among the general public to stimulate the bone formation phase. To combine VEGF with stem cells to (theoretically) provide the optimal environment for bone growth could enhance its effect.

Multiple articles have been published each year in an attempt to find a solution for better and faster bone healing. In this thesis, we attempted to test the need for VEGF by focusing only on bone growth along with different release methods that are possible to use in both animals as well as in the clinical setting. To date, it has been 30 years since the first efforts in this area, and very few bone

tissue engineering techniques have actually translated into clinical practice, while none have become part of standard care in regenerative medicine (7).

Purpose/hypothesis

General purpose

The general purpose of this thesis is to test angiogenic factors combined with MSC on different types of bone healing in well-tested designs and different animals. The designs should provide reproducible and translational methodologies to learn more about the combing and solely effect of enhancing angiogenesis on bone formation.

This thesis was devolved continuously while focusing on the most relevant perspectives due to our obtained results. As such, we used the results from each study to be incorporated and developed for the next design.

The focus throughout this thesis will be on the osteogenic effect and evaluation of new bone development using our methods and dosages.

General hypothesis

It is possible to enhance ectopic and endochondral bone regeneration in different animal models with the use of mesenchymal stem cells and/or VEGF when compared to osteogenic factors only or allograft.

Study 1: To investigate the most efficient time point for VEGF stimulation combined with MSCs on ectopic bone formation in severe combined immunodeficient (SCID) mice compared to only MSC treatment.

Hypothesis: VEGF- and MSC-seeded HA would have an additive effect on the stimulation of bone formation in the early generative phase when compared to MSC only.

Study 2: To determine the optimal dose of VEGF combined with MSCs *in vivo* in a bilateral distal femur implant gap model *in vitro* and *in vivo* in normal sheep for 12 weeks to determine new bone formation compared to allograft.

Hypothesis: The effect of autologous MSCs on bone formation has the potential to be stimulated by additional VEGF coating when compared to the gold standard of allograft.

Study 3: To collect all existing *in vivo* results on the solitary use of VEGF for bone growth compared to control (evaluated by new bone volume / total volume (BV/TV)) and to evaluate whether these results indicate any promising progress towards release methods and dosages that could be applied in a focused experimental design and translated into human clinical use.

Hypothesis: Solitary VEGF stimulation in current *in vivo* literature will show a pattern and method for human applications in bone formation.

Methodology

Animals

The surgical design was performed under the consideration of having several implants per animal that should be used as little as possible but still comply with the power calculation. This was done according to *in vitro* and pilot studies as well as developments in the methodology and design models.

The ectopic mouse model design allowed us to assess the effect of VEGF dosages, combination to MSC and methods on the bone-forming capabilities with the focus of time administration. There were several pouches in each animal, which were created using a mildly invasive technique. Furthermore, since the breed of mouse was an SCID, we did not have any immunological reactions when transferring sheep mesenchymal stem cells into the host mouse. Small animal models also provide the possibility of minimising the number of biases and provide consistency due to the animals being bred for a purpose. All mice had regular cages with the same humidity, temperature, light and amount of food.
Large animal models are well known to be a better representative for correlation into human trials than rodent models. This translational research is usually the pre-step in research to provide sufficient evidence for human trials. The disadvantage of large animal models is that they are rarely bred for research and thus often do not have the same genetic pool. Compared to the small animal models, they are also more difficult to baseline (regarding age, weight, housing, etc.) as per human trials. A solution for these biases involves having the control group and intervention within the same animal. This design is used to minimise individual differences and thereby exclude the need for a baseline on general parameters.

The sheep model is commonly used within the field of bone research (71). However, there is disagreement regarding which model is the best representative to illustrate the effect on human bone. Elderly sheep are used due to their similarity in size to humans, while their osteoid volume and mineral apposition rate serve as a good model for the testing of bone implant materials (72). Previous studies have suggested that the remodelling in sheep occurs faster than in humans (71). In our sheep designs, we tested the defect size used in this thesis in normal bone structure after 12 weeks with no intervention or implant. This resulted in no bone growth to bridge the gap, characterising its nature as a critical size defect (CSD) (Figure 1). However, each sheep contained all intervention and control groups for comparison within the same animal, so even though the percentage of bone formation cannot be compared directly to human tissue, the bone forming effect between intervention groups and control still serves as a reliable indication.

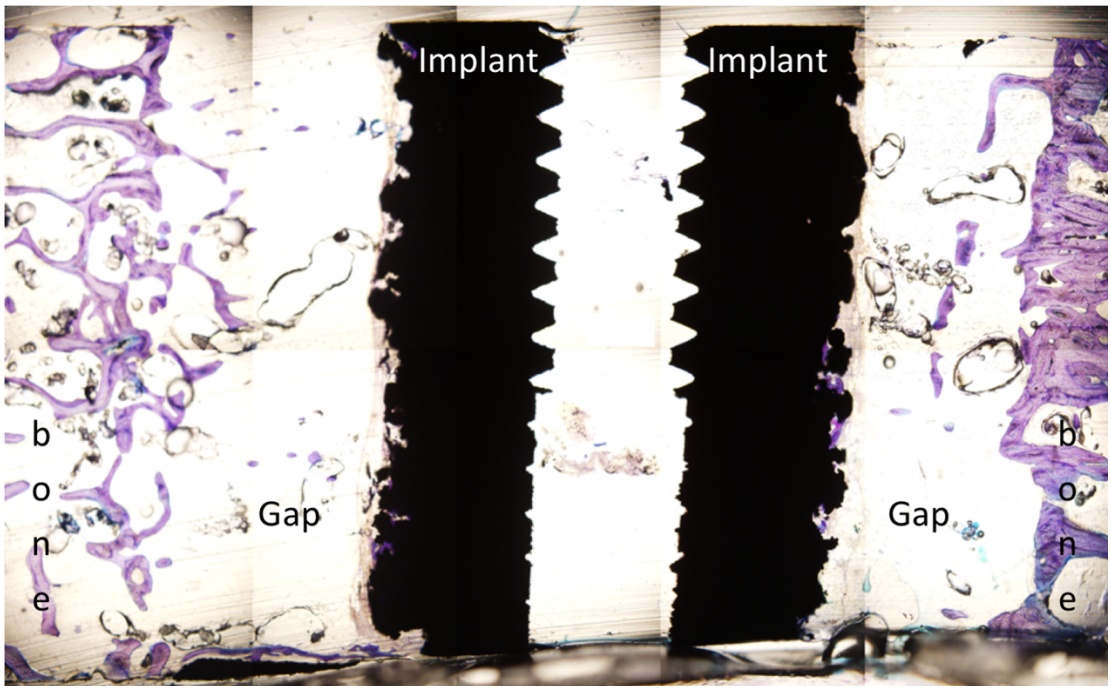


Figure 1: Illustration of an empty implant in a normal sheep after 12 weeks of observation stained with toluidine blue O

Implant design

This thesis used one type of implant bone block as a substitute (Study 1) and one type of regular implant (Study 2) with a focus on different locations and models. One type focused on the bone-forming capabilities in the ectopic formation (Study 1), while the other methods were implanted in the trabecular bone structure in the femur bone with the general focus being on osteointegration and

osteogenic function (Study 2). This was done to mimic different clinically relevant situations using an evaluation period of 8 weeks for the small animal model and a 12-week evaluation period for the large animal model, with a focus on posterior lumbar fusion in the ectopic design and a greater focused on trauma in the implant model.

Ectopic implant bone block in mouse (Study 1)

Three to four implants were subcutaneously inserted in pouches on the backs of mice (Figure 2). A 1ml syringe was filled with 40mg of pure HA granules seeded with MSC combined with a VEGF pellet defined as the implant bone block. This implant bone block was injected through the syringe and externally fixated for correct placement. The material had a diameter of approximately 12mm, depending on the placement of each granule, with an expected size increase due to edema of up to 50%.

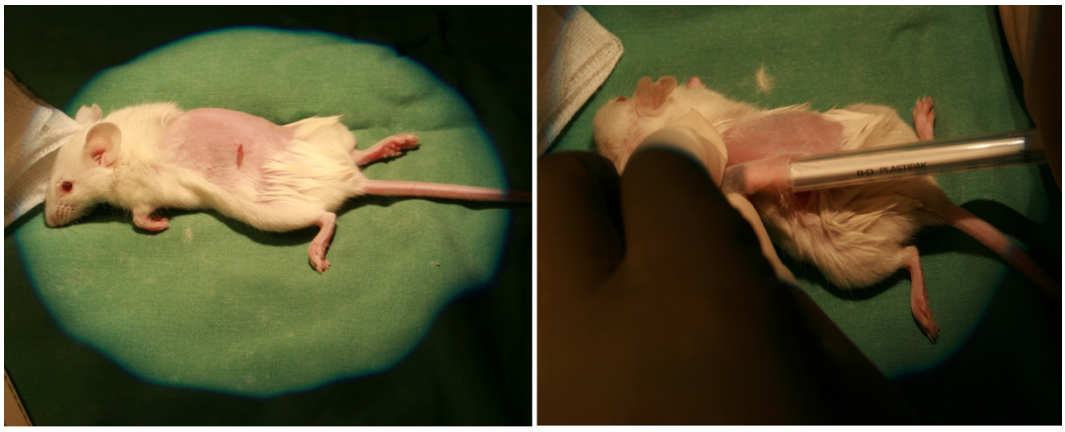


Figure 2: Illustration of incision and placement of the implant-bone-block by 1ml sterile syringe

Femoral implant gap model with titanium implants in sheep (Study 2)

Plasma-sprayed implants (Ti-6A1-4V) were placed in the medial and lateral side of each distal femur condyle in sheep. The implant and top washer consist of 90% titanium, 6% aluminium and 4% vanadium, with a general size of 10mm x 12 mm (Biomet Inc, Warsaw IN, USA, Figure 3). In the femoral implant gap model, four implants were inserted into each sheep femur on both the lateral and medial condyles. This allowed the design to have several interventions and controls within the same animal.

The drill (10mm diameter) was placed 14mm into the bone while considering the trabecular bone structure. Firstly, the footplate and neck were placed in the bone defect, and the gap was then filled with different materials according to relevant groups. Finally, the top washer fixated the material within the gap around the implant and closed the defect. The implant was either a regular implant or an implant coated with the VEGF growth factor and kept at -20 degrees before insertion. The gap was filled with HA seeded with MSCs or allograft.

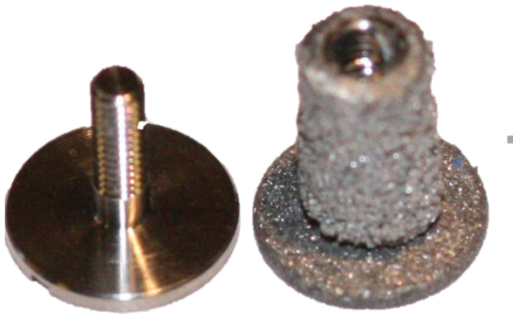


Figure 3: *Ti-6A1-4V implant 10x12mm illustrated separated into the top washer and footplate including the neck of the implant.*

Study design

Study 1:

A total of 28 SCID Jackson mice (NOD.CB17-Prkdcscid/J) were included in the study. The intervention involved a combination of MSCs and VEGF at different time points to focus on bone formation. The amount of MSC used, the dosage of VEGF, the time of observation and the strain of animal were tested in two separate pilot studies.

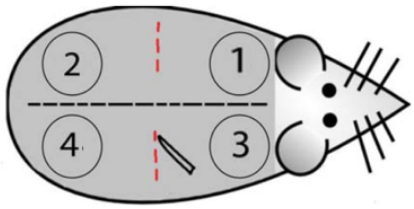


Figure 4: Illustration of the position of the s.c. implant-bone-blocks in the mouse model

Pilot study 1

Had the focus points

1. The breed
2. The amount of MSC

3. The best time of observation

The pilot study had the following groups

	MSC	Observation time
1 mouse Taconic NOD/MrkT – 4 implants	1 x 10^5	7 weeks
1 mouse Taconic NOD/MrkT – 4 implants	2.5 x 10^5	8 weeks
1 mouse Taconic NOD/MrkT – 4 implants	5 x 10^5	8 weeks
1 mouse Taconic NOD/MrkT – 4 implants	1 x 10^6	9 weeks
1 mouse Jackson Prkdc,SCID/NcrCrl – 4 implants	1 x 10^5	7 weeks
1 mouse Jackson Prkdc,SCID/NcrCrl – 4 implants	2.5 x 10^5	8 weeks
1 mouse Jackson Prkdc,SCID/NcrCrl – 4 implants	5 x 10^5	8 weeks
1 mouse Jackson Prkdc,SCID/NcrCrl – 4 implants	1 x 10^6	9 weeks

Pilot study 2

Had the focus point

1. Optimal VEGF dosage.

Dosages on the toxic threshold for the stimulation of VEGF remain lacking in the literature. A study on rats had administrated 47ng of VEGF by a bolus intracerebroventricularly (49), while another mentioned associations with central nerve system (CNS) diseases such as Alzheimer's with elevated thrombin levels at 500ng/day in a rat model (73). Therefore, we chose a much lower dose in a smaller animal mode to ensure that we did not reach this dosage threshold.

	MSC	VEGF	Observation time
1 mouse Jackson Prkdc,SCID/NcrCrl – 4 implants	5 x 10^5	8ng release/day	8 weeks

1 mouse Jackson Prkdc,SCID/NcrCrl – 4 implants	5 x 10^5	16ng release/day	8 weeks
1 mouse Jackson Prkdc,SCID/NcrCrl – 4 implants	5 x 10^5	24ng release/day	8 weeks
1 mouse Jackson Prkdc,SCID/NcrCrl – 4 implants	5 x 10^5	Placebo pellet	8 weeks

The conclusion of both pilot studies indicated the best results for bone formation by histology with H&E staining for Jackson Prkdc and SCID/NcrCrl with the use of 5 x 10^5 MSCs with 8ng VEGF release/day for 8 weeks. These results were incorporated into the primary study.

Primary study:

Breed: Jackson Prkdc,SCID/NcrCrl	**Mesenchymal Stem Cells**	**Vascular Endothelial Growth factor**	**Time point for release**
4 mice – 3 implants per mouse	5 x 10^5	8ng release/day	1-7 days
4 mice – 3 implants per mouse	5 x 10^5	8ng release/day	1-14 days
4 mice – 3 implants per mouse	5 x 10^5	8ng release/day	1-21 days
4 mice – 3 implants per mouse	5 x 10^5	8ng release/day	1-42 days
4 mice – 3 implants per mouse	5 x 10^5	8ng release/day	7-14 days
4 mice – 3 implants per mouse	5 x 10^5	8ng release/day	21-42 days
4 mice – 3 implants per mouse	5 x 10^5	-	-

The different time points for VEGF stimulation were chosen due to the natural rise of VEGF in fractures within weeks 2–3 in both human (74) and rodent models (75,76). The MSC were seeded

onto pure hydroxyapatite (HA) granules for a local and isolated effect. The VEGF release was then made from verified pellets by the Innovative Research of America (IRA), which were inserted next to the implant bone block consisting of MSCs and HA. The control group contained the same amount of MSC but without any VEGF stimulation.

Each mouse had three pouches of this combination due to issues in the pilot study involving the migration of HA granules. The three pouches were from the same group and not mixed. This means each mouse contained either only intervention or control. This was a necessity in the design due primarily to pellet release so that inserted pellets did not affect other groups; moreover, this was also due to blood serum samples that had to be representative of only one group. Yet, the parameters of the serum samples did not have a strong power calculation due to only four mice being included in each sample group. Our focus on the systemic measure was to show the absence of a systemic rise, while the values between each group were secondary.

Study 2:

In vitro pilot study

Had the focus points

1; If the combination of poly-d,l-lactic acid (PDLLA) coating for the release of VEGF had any negative influence on the differentiation of MSCs.

2; If any difference could be illustrated using either normal or osteoporotic cells.

A total of 0.5×10^4 MSCs were used separately or combined with VEGF PDLLA-coated implants for a release of 100ng/day. Furthermore, we used MSCs in both normal and verified osteoporotic sheep to observe any difference in osteogenic capabilities.

The amount of MSCs was chosen based on the results acquired from the *in vivo* study in mice where the certified release of the VEGF protein via pellets was used. These dosages thus provided the opportunity for an *in vivo-in vitro* comparison of the release methods and their osteogenic effect.

The MSCs had the same aspiration procedure and differentiation protocol from the sheep model (as mentioned in Study 1). The osteoporotic cells were thawed 4 days before the normal cells due to our experience of faster seeding growth. After 11 days for the normal MSC and 7 days for the OP MSC, the cells were seeded until 100% confluent, which was achieved for all cells within 48 hours. The VEGF followed the coating procedure on titanium implants and was added after the cells were

defined as confluent as per the *in vivo* designs. The groups were focused on the initiation of the model into the large animal model. Staining was conducted after 18 days

	Normal MSC	VEGF on titanium neck implant
Group A1 –	0.5 x 10^4	100ng release/day
Group A2 –	0.5 x 10^4	-
Group A3 –	-	100ng release/day

	Osteoporotic MSC	VEGF on titanium neck implant
Group B1 –	0.5 x 10^4	100ng release/day
Group B2 –	0.5 x 10^4	-
Group B3 –	-	100ng release/day

To visualise the osteogenic effect and differentiation, the composed product and scaffold were stained for extracellular calcium deposits with alizarin red and alkaline phosphatase. This process illustrated the differentiation of MSCs into the osteogenic lineage as well as the effect of VEGF and the coating on the differentiation process. The staining protocol from PromoCell was used and evaluated by macroscopic imaging (77). Notably, alkaline phosphatase staining did not present a visual conclusion in the outcome with normal cells (Figure 5).

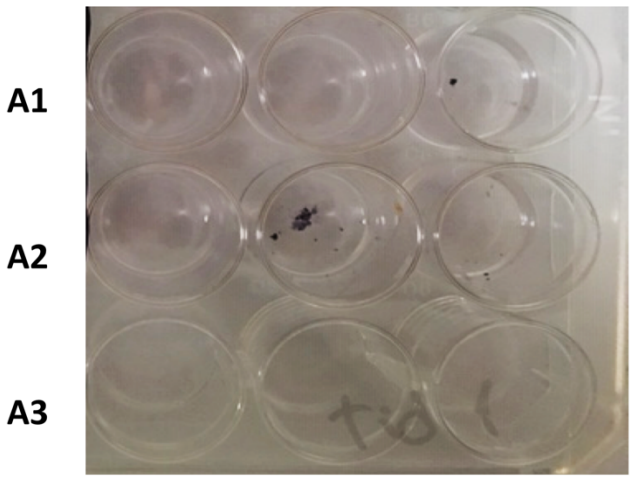
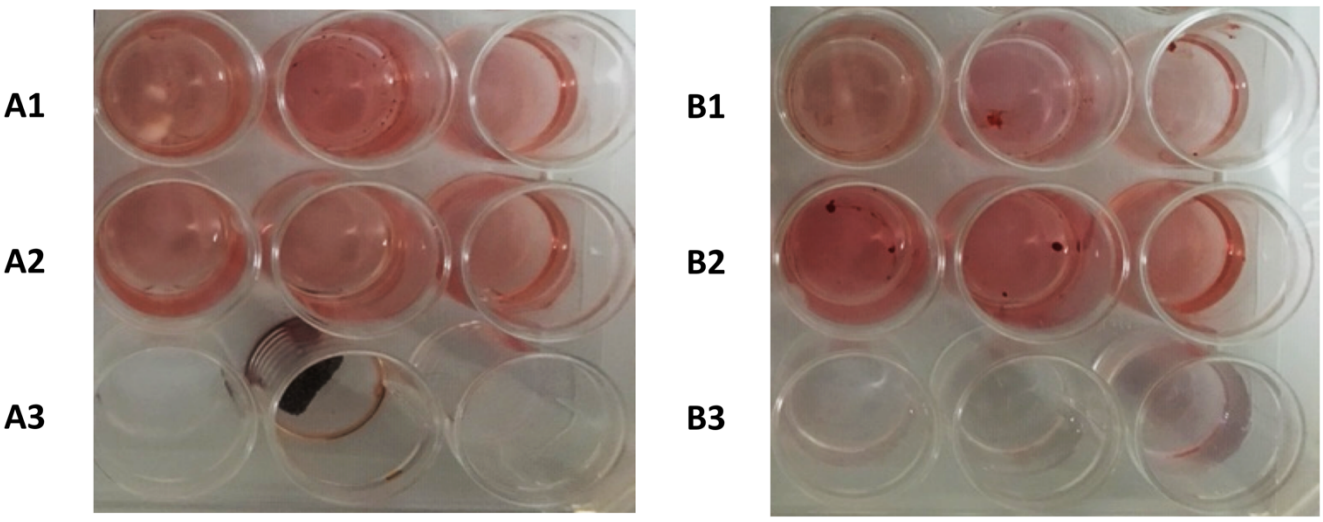


Figure 5: Illustration of the in vitro study with alkaline phosphatase staining

Furthermore, alizarin red staining showed no difference between the MSCs alone or in combination with VEGF in normal sheep (Figure 6: A1, A2). In the OP cells, there was a slight indication that

MSC alone seem to have larger calcium deposits in the osteogenic culture (Figure 6: B1). The VEGF only groups had unstained to slightly stained samples (Figure 6: A3, B3).



A: Normal Cells B: Osteoporotic cells

Figure 6: Illustration of the in vivo study with alizarin red staining

The pilot study indicated that the combined treatment with VEGF and PDLLA coating materials on the MSCs seems to have no osteogenic influence compared to MSCs alone. Furthermore, no difference was illustrated between normal or osteoporotic cells in the culture.

In vivo pilot study

The *in vivo* pilot study focused on:
1: The amount of MSCs in combination with a low dose of VEGF compared to MSCs alone.
2: Validation of the amount of HA granules within the gap in order to achieve optimal design and osteogenesis.

The *in vivo* pilot study included eight Texel/Gotland sheep with four implants in each sheep. The intervention was performed in normal bone structure.

The amount of MSCs used was determined by:

1: A previously unpublished study by our group that had negative results with the use of more than 5 million MSCs in this design.
2: The ratio between HA and MSCs in Study 1 being 1mg HA per 12500 MSCs.
3: The results from the *in vitro* pilot study.

The low dose of VEGF was based on the use of 8ng VEGF release/day in Study 1. The amount of HA granules was based on the calculation of total gap volume divided by the average size of one granule.

	HA	**MSC**	**VEGF**
Group 1 – 8 implants	240mg	1 x 10^6	10ng release/day
Group 2 – 8 implants	240mg	3 x 10^6	-
Group 3 – 8 implants	240mg	3 x 10^6	10ng release/day
Group 4 – 8 implants	240mg	5 x 10^6	10ng release/day

The pilot study indicated the use of 3 x 10^6 MSCs in combination with VEGF had the best result on bone formation, as evaluated by stereology. Notably, 240mg of HA granules could only fill 50% of a gap. As such, the actual study was designed based on these results. Total gap size can be calculated by the area of the neck of the implant withdrawn from the total area of the implant (Figure 3):

$$(3.14\ x\ (5mm)2\ x\ 10mm - 3.14\ x\ (3mm)2\ x\ 10mm) = 502mm3$$

This provides a total of 0.502ml. However, the granules have such high variation in size that we found it difficult to calculate the exact amount to be used in gaps.

Actual study in normal sheep

	HA	**MSC**	**VEGF**
Group 1 – 8 implants	500mg	3 x 10^6	10ng release/day
Group 2 – 8 implants	500mg	3 x 10^6	100ng release/day
Group 3 – 8 implants	500mg	3 x 10^6	500ng release/day
Group 4 – 8 implants	500mg	Allograft	-

To correlate the HA amount from the pilot study, standardised tubes with 500mg HA (Fin-Ceramica, Faenza, Italy) were used. This resulted in less variation in size and thus a better structural fit within the gap model.

The VEGF dosages were chosen according to:
1: The use of 8mg VEGF release/day in a mouse model (Study 1).
2: An *in vitro* study that used 100ng VEGF release/day.
3: The existing literature

To our knowledge, no existing study has been performed on the systemic toxicities of VEGF in sheep. A 12-week observation period was chosen, which is assumed to be an intermediate time period for bone regeneration in the sheep model (78). Furthermore, the recommended observation in sheep and goat models suggest a timeframe of 12 weeks in implant fixation studies (79,80).

Study 3

This systemic review article was built around the basic principles and reporting of methods used by well-defined protocols from Sycle for reviewing animal intervention studies, which was adapted from the Risk of Bias tools developed by Cochrane (81) and the check-list schedule from CAMARADES for evaluating the quality of experimental studies (82). Articles were evaluated by modified versions of these to specify their usage toward the hypothesis of the study. The protocol for this study strictly followed the version from Sycle for developing high-quality animal study systematic reviews.

The focus of this study was to obtain results for the use of only VEGF stimulation *in vivo* for bone growth, without any other stimulants. In 2009, a systematic review highlighted the great potential for VEGF in bone research (44). Thus, our search included the 2009–2019 period to evaluate the last 10 years of progress towards clinical practice. However, the first study in this systematic review was from 1 January 2007; therefore, the search in this study was initiated from this date. The primary outcome was bone formation by BV/TV, preferably evaluated by histology. The secondary outcome was the release method and dosage affecting the results.

The search was performed using the following search string:

(vegf or "vascular endothelial growth factor") and (osteogenic or "bone formation" or "bone regeneration").

Studies were then filtered for full text only, human or animal studies, and publication date from 1 January 2007 to 01 July 2019.

The inclusion of articles was conducted in three phases. Phase 1; Involved evaluating titles and abstracts as well as the removal of duplicates, which was completed by the 1st author in separated time frames with at least 3 months in between. Phase 2; Involved the evaluation of the full texts of articles with the confirmation of two researchers (first and second author) based on inclusion criteria. The final phase involved a quality assessment/risk of bias and schedule of each included study. This phase was analysed by the first and second author.

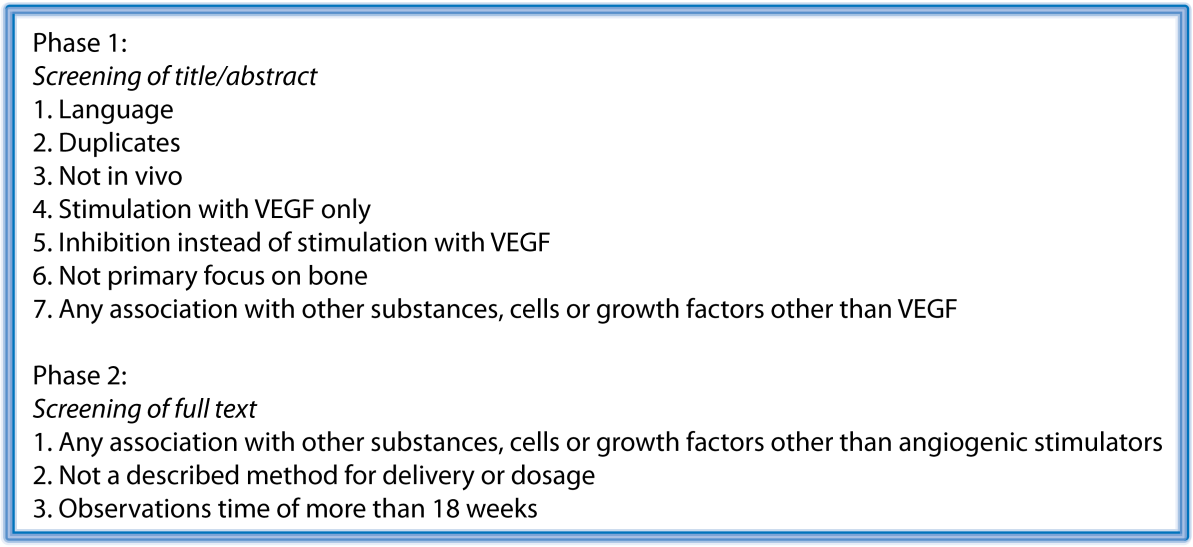


Figure 7: illustration of each phase for the inclusion of articles

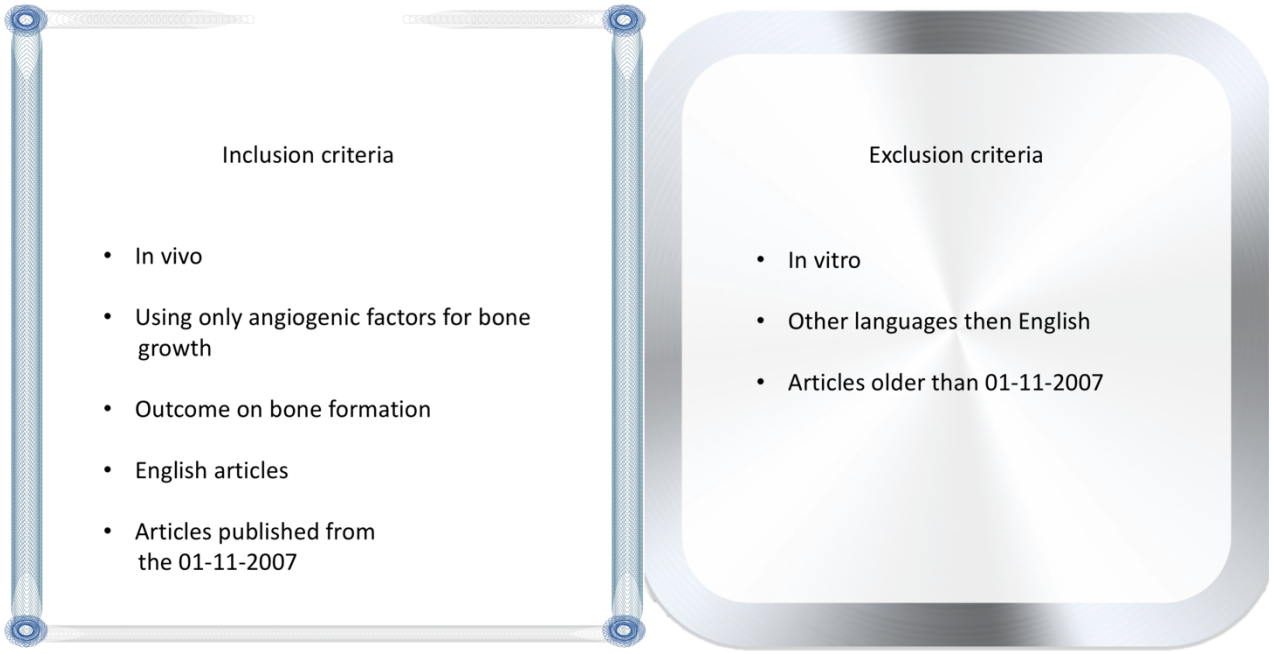


Figure 8: General inclusion and exclusion criteria

A minimum of six articles involving well-defined bone formation was chosen as the minimum criteria for statistical purposes. It was further required that quantitative data were available.

Risk of bias was assessed using a modified quality score by CAMARADES for systemic reviews in experimental animal studies (Table 1, (82)).

Characteristics of Included Studies

Publication	Year	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	Score	Ref
Sun	1998	X	X	X		X	X					5	11
Ayoub	1999	X	X				X			X		4	12, 21
Ogilvy	1999						Nk					0	23
Mokudai	2000	X	X	X	X	X	X			X		7	13, 22
Sakakibara	2000	X	X		X		X			X		5	14
$Maynard_1$	2001	X	X				X			X		4	15
$Maynard_2$	2001						Nk					0	27
Shen	2001						Nk					0	25
Ayoub	2002	X	X				X			X		4	16, 26
Chang	2002	X					X			X		3	17
Sakakibara	2002	X	X			X	X	X		X		6	18
Yang	2002	X								X		2	19
Sadanaga-Akiyoshi	2003	X	X	X			X	X		X		6	20, 28
Sarhan	2003	X	X				X			X		4	24, 29

Studies fulfilling the criteria of: (1) peer reviewed publication; (2) control of temperature; (3) random allocation to treatment or control; (4) blinded induction of ischemia; (5) blinded assessment of outcome; (6) use of anesthetic without significant intrinsic neuroprotective activity; (7) animal model (aged, diabetic, or hypertensive); (8) sample size calculation; (9) compliance with animal welfare regulations; and (10) statement of potential conflict of interests. Also see supplementary material.

Ref indicates references; Nk, not known.

Table 1: The modified risk of bias tool from CAMARADES (82) *from which study items were constructed*

Three items from Table 1 did not have any purpose in this systematic review and were removed:

2) Control of temperature.

4) Blinded induction of ischaemia.

6) Use of anaesthetic without significant intrinsic neuroprotective activity.

Moreover, the following two criteria were added to focus on the aim of our study:

1) Control group presented.

2) VEGF dose justified.

This resulted in a total of nine criteria for the total risk of bias assessment:

(1) peer-reviewed journal; (2) control group; (3) randomisation; (4) VEGF dose justified; (5) blinding; (6) details on the animal model; (7) sample size calculation; (8) compliant with ethics; and

(9) no conflicts of interest.

An overview schedule was created to gather all information from each included article. This consisted of the subject: (1) animal; (2) type of defect; (3) control group; (4) VEGF type; (5) release method; (6) release rate; (7) VEGF dosage; (8) time of evaluation (TOE); (9) results; (10) specific BV/TV; (11) quality score/risk of bias.

An assessment and comparison of the studies was made according to described procedures and statistical results. However, it was difficult to acquire such comparison between the studies due to either a lack of details or the use of many different methods in provided descriptions. Therefore, a heterogeneity assessment could not be performed.

Release methods for vascular endothelial growth factor

The IRA pellets and implant coatings had the purpose of delaying the release of VEGF around the implant bone block, thereby securing local distribution within the area.

The pellets consisted of cholesterol, cellulose, lactose, phosphates and stearates (IRA) with degradable properties. Each pellet compound contained recombinant human rhVEGF165 (293-VE, R&D systems, Bio-Techne) along with BSA at a ratio of 1:50. This carrier protein is frequently used as a stabiliser for other solubilised proteins and binds water, salts, vitamins, fatty acids, hormones and carries these bound components between tissues and cells, thereby stabilising the binding of the protein (68,69). The pellets contained 8ng VEGF/day times the number of release days according to the intervention group (69).

The coating method was inspired by previous substitute materials used by our group with the femoral gap design and consisted of biodegradable Poly(D,L-Lactide) (PDLLA) (83,84). However, this design is made by coating the neck of the titanium implant and is not localised on any substitute. The coating consisted of the firm material PDLLA mixed with liquid solvents. The neck of the titanium implant was coated twice until it could be macroscopically visualised to cover the sand-dusted surface area. The implants were stored at -20^{o} until surgery.

The release profile was tested by a Bradford protein assay measurement. A total of 4 implants were then coated and placed in phosphate-buffered saline (PBS) for 4 weeks. The implants were removed at the 1st, 2nd, 3rd and 4th week to be analysed for protein in the remaining fluid. While protein could

be localised by the assay in the first 3 weeks, no protein within the range of the kit could be measured in the last week. This gave an indication of a release profile spanning the first 3 weeks post-surgery. Previous studies have noted the molecular weight of the growth factor as being a factor for delivery through coatings. For future reference, VEGF has a weight of 7.5 kDa (kilodalton). All substances in this coating have been approved by the food and drug administration (FDA) for individual use.

The dosage calculation was thus based on a full release of the total VEGF in the first 3 weeks after surgery, and the total dosage was divided by 21 for a daily release. Since the product of rhVEGF165 was in combination with BSA in a ratio of 1:50, when coating one implant with the secretion of 100ng VEGF release/day, the total dosage of 100ng x 21 days x 50 BSA was added to the mixture for a total amount of 105.000ng/0.105mg rhVEGF165 per implant. For 10ng VEGF/day, it was 10.500ng/0.0105mg and 500ng VEGF/day = 525.000ng/0.525mg.

After the coating was performed, the implants were shipped at -20^{o} and sterilised with gamma irradiation by Synergy Health Radeberg GmbH, STERIS Germany prior to surgery.

Surgical procedure

The surgical procedures for Studies 1 and 2 were performed at the Biomedical Laboratory, University of Southern Denmark.

Studies 1 and 2 - aspiration of bone marrow

Bone marrow was aspirated from female sheep between 5–8 years of age, with weights of 53–77kg (mean=67kg). This procedure was performed under sterile conditions while the sheep were fully anaesthetised. Local analgesia of 5ml of lidocaine (20mg/ml FarmaPlus, UK) was given in the area around the penetration site. Two incisions approximately 10 cm lateral from the spina iliac posterior superior edge were made in each side of the sheep. Then, 3–4ml of bone marrow was aspirated by a bone marrow aspiration needle 13ga x 2.1/2in (Angiotech, Vancouver, Canada). The entire process was performed under the supervision of laboratory technicians.

The extracted bone marrow was cultivated for a maximum of two passages (usually 14 days).
In Study 1: MSCs were mixed with 40mg(+/-1mg) of HA and 100ul 10% fetal bovine serum (FBS) MEM medium was added with a syringe and packed overnight at 37^{o}C, 5% CO_2.

In Study 2: MSCs were seeded on 500mg HA in a 5 ml syringe using the same method.

Surgery severe combined immunodeficient (SCID) mice

The surgical procedure followed an existing protocol from the Department of Endocrinology, Odense University Hospital (85) that was created by one of the co-authors of this study. Together with the rhVEGF165 pellets (IRA, Sarasota, FL, USA), the MSC-seeded HA was implanted subcutaneously (s.c.) into the SCID mice (Figure 10, day 0).
The middle region of the back was shaved at each side. Ethanol was used to sterilise the skin and a horizontal incision of 1–1.5cm was created bilaterally. An empty 1 ml syringe separated the outer skin from the fascia. The 1ml syringe containing the HA coated with MSC and the VEGF pellet could be inserted s.c. without resistance and placed into the pouches. HA placement was essential to the migration process and this was fixated externally, approximately 1.5cm from the incision site. The procedure was repeated for all three to four pouches.

The operations were completed between 12 a.m. and 6 p.m. in the Special Care Pathogen-free (SPF) Department at the Biomedical Laboratory Facility, University of Southern Denmark. The analgesia followed house protocol, with 0.1ml ketamine /1g of bodyweight intraperitoneal (IP) being supplemented with an isoflurane (4%) vaporiser chamber with supplementary oxygen. The pain treatment was Temgesic 0.3mg/ml mixed with 10ml of water pr. 1ml. every 6–8 hours (s.c.) for the first two days after the operation (69).

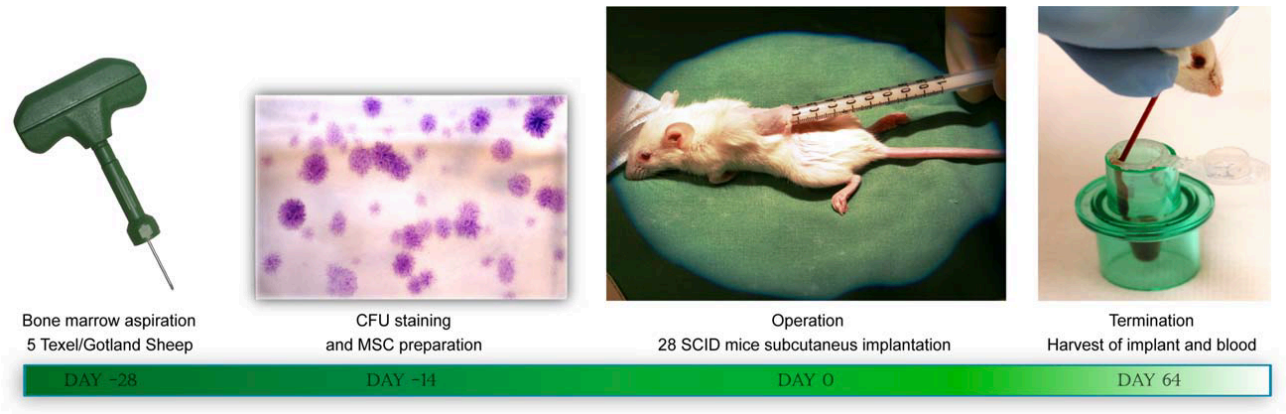


Figure 10: Illustration of procedures performed in study 1. Operation method is illustrated at Day 0

Surgery sheep distal femur implant gap model

The sheep fasted for 24h prior to any invasive procedures. They were then placed on an operating table with fixated legs on either the left or right side for the exploration of the medial or lateral distal part of the femur bone. The skin was shaved and disinfected with ethanol (70%) and iodine (Kruuse, Langeskov, Denmark). An incision divided the outer skin and the fascia with the use of a scalpel and vet monopolar diathermy, while the muscle tissue was separated until the periosteal surface was isolated (Figure 11, T4). A high-speed drill created a 14-mm deep cylindrical hole with a circumference of 10 mm in the same size as the titanium implant. After the drilling process, excess bone particles were rinsed with saline to make sufficient room and secure the proper placement of the implant (Figure 13, B). The gap was filled with either HA or allograft, and the top washer secured the isolation of the material within the gap. The wound was sutured in three layers and sprayed with Wound Plast with Tar (Kruuse, Langeskov, Denmark). The procedure was repeated for both sides as well as for the opposite femur using existing protocols (27).

The initial analgesics were 0.01ml/kg Rompun vet (20mg/ml Bayer Healthcare, Berlin, Germany) and Propofol (10mg/ml B. Braun, Frederiksberg, Denmark). After intubation and during surgery, isoflurane 2% kept the sedation consistent. Pain supplement was Temgesic every 6 hours for the first 24 hours after surgery. The antibiotic used was Curamox Prolangatum vet (150mg/ml, Boehringer Ingelheim, Copenhagen, Denmark) intramuscularly (i.m.) for the first 3 days postoperatively.

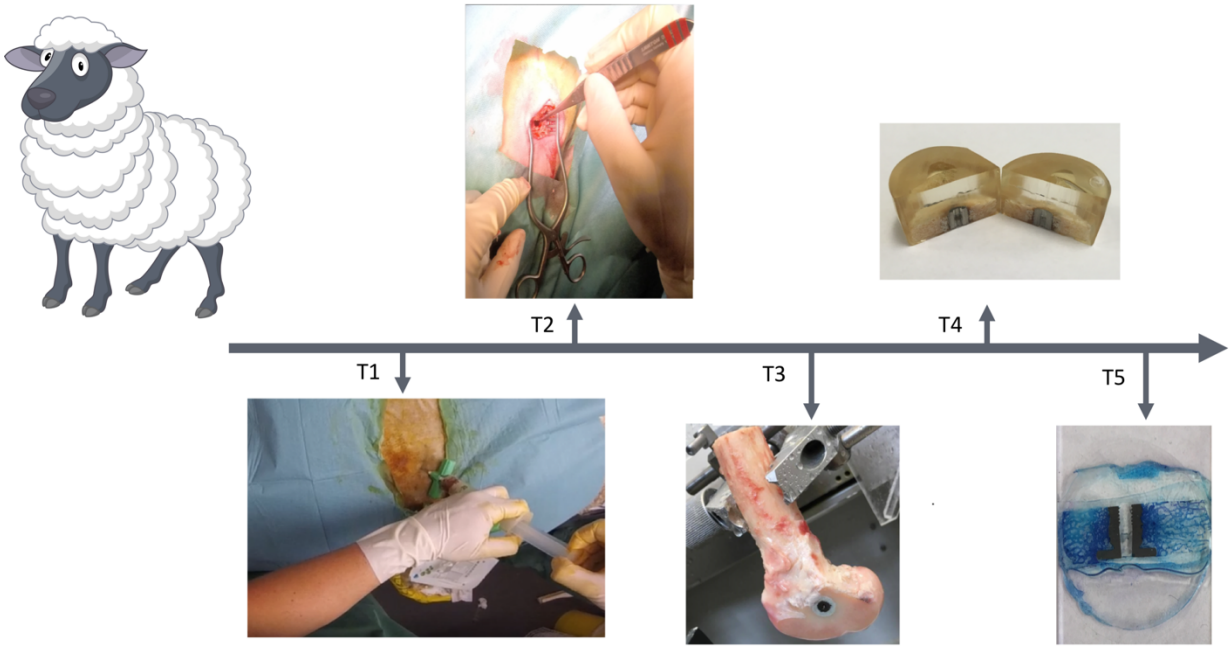


Figure 11: Illustration of the order of intervention in study 2: T1 (week 0): Aspiration of bone marrow for cultivation of MSC. T2 (week 4): Surgery femoral implant gap model. T3 (week 16): preparation of samples for MTS and embedding. T4 (week 28) samples embedded and ready for sectioning. T5 (week 29) samples stained for evaluation.

Animals and housing

The pilot study on mice used Taconic NOD/MrkT (Cologne, Germany) and NOD-scid SCID mice from The Jackson Laboratory (NOD.CB17-Prkdc^{scid}/J, Maine, USA). Only NOD.CB17-Prkdc^{scid}/J mice were used in the primary study.

The mice were housed in the Biomedical Laboratory Facility in the Pathogen-free Special Care Unit at the University of Southern Denmark and were acclimated for 1 week prior to surgery. The environment was controlled for temperature (21–28C) and humidity (40–60%), with lights being on between 6 a.m. and 6 p.m. The mice were housed individually in ventilated cages (IVC) with sawdust flooring and bedding material. Then, 4–5 days after surgical intervention, 3–4 mice were gathered into each cage. They had access to sterile water and Altromin total pathogen-free diet and were observed daily by either the animal technicians or the researcher for changes in behaviour or signs of discomfort (69).

The Texas/Gotland sheep breed was chosen as the large animal model. All sheep were housed at an external farm for large research animals under the management of the Biomedical Laboratory Facility, University of Southern Denmark. The environment is designed for research purposes with flat surface for fragile bone structures. The sheep had the possibility to enter an outdoor area during the summer while being housed indoors with hay, water *ad libitum* and regular dietary chips for sheep in calculated dosages. While the temperature was not regulated, animals were observed daily for signs of discomfort and coats to keep temperature were applied during the winter. One month prior to any invasive procedures, the acclimatisation period was initiated according to animal guidelines. After each procedure was performed on the animals, they were housed at the Biomedical Laboratory for 5 days for frequent observation and pain supplement before being transported to the external farm.

All the institutional and national guidelines for the welfare and use of small and large animals were followed and comply with Animal Research: Reporting of *In vivo* Experiments (ARRIVE) guidelines. All animal research procedures were approved by the Danish Animal Experiments Inspectorate (no. 2012-15-2934- 00704).

Cultivation of bone marrow cells

Following aspiration, 4ml of bone marrow was transferred into a 50ml falcon pipe containing 4ml of minimum essential medium alpha (MEMA, alphaMEMA, Life Technologies, Europe BV, Denmark) and 1ml heparin to avoid clotting. The liquid was stored in a normal fridge at +4ºC.

Bone marrow was then diluted with PBS (Dulbeccos, Life Technologies Europe BV) at 1:1, followed by 13ml of ficoll being added. This was then centrifuged for 30 minutes to separate the blood components. Between the ficoll and the lymphocytes, a middle layer was exposed for collection and mixing with 50ml of PBS.
The cells were then washed in MEM and centrifuged, resuspended in 10ml of preheated (37ºC) alpha MEM, FBS (Sigma-Aldrich) and penicillin streptomycin glutamate (PSG, Life Technologies Europe BV). Then, 100uL of cell suspension was used for the cell count and $1x10^5$ for the colony-forming unit (CFU) cultivation. The cell count was performed manually using a counting plate and a microscope.

Colony-forming unit (CFU) cultivation was performed over 14 days with a constant temperature of 37ºC in an incubation locker. After 14 days, the medium was washed with PBS and 4% formaldehyde was added and incubated for 5 minutes to fixate the cells. The formaldehyde was removed and washed twice with PBS. Then, the cells were incubated for 30–60 minutes and coloured with crystal violet blue for calculation. The crystal violet blue was then aspirated and rinsed with water. By counting plates, we evaluated how many of our $1x10^5$ cells had osteogenic potential. Notably, the minimal amount for the use of CFU values is stated to be higher than $7.5x10x^3$ (86).

The subculturing of cells was performed at 80–90% confluency. They were then washed with 10ml PBS and 3ml of trypsin-ethylenediaminetetraacetic acid (EDTA, 0.05%; Life Technologies Europe BV) was added. Therefore, they were incubated for 3 minutes to make the cells loosen the plate. Then, 7ml of MEM was added and centrifugated at 200g for 5 minutes, which isolated the supernatant. The next step involved 10ml of MEM being added and 6–10ul for the manual counting procedure. The total number of cells/ml was divided into separate tubes of $5x10^5$ cells each.

After subculturing, the cells could be seeded on HA for surgery or cryopreserved for later use. Cryopreservation involved a combination of 2ml FBS, 100ul 1% penicillin streptomycin, 1ml

dimethyl sulfoxide (DMSO) and 7ml MEM for a total of 10ml of solution. Previous subcultured cells were centrifuged and the cell number was diluted with the freezing medium. Lastly, 1ml could be stored in each tube containing $1x10^6$ cells at -80ºC (69).

Allograft

Allograft bone was gathered from the proximal and distal femurs of five healthy sheep not used in other studies. The sampling only consisted of the trabecular bone and was pooled in a mixture from all sheep. Under sterile procedures and materials, the bones were stripped for any soft tissue, and the trabecular bone structure was divided into chips using a manual bone mill (Ossano Scandinavia ApS) in the Biomedical Laboratory, University of Southern Denmark. The allograft chips were filled in sterile glass, bagged and stored according to protocol at -80°C. Before use, the allograft would be thawed for 30–45 minutes.

Coloring forming unit (CFU)

The CFUs were made according to existing protocols (87). These provided an indication of how many stem cells had the capability to differentiate into the osteogenic lineage. The purpose of plate counting is to estimate the number of cells present in the microscope based on their ability to give rise to colonies under chosen conditions of nutrient medium, temperature and time. The theory is that one viable cell can give rise to a colony through replication, which thereby provides an estimate of the osteogenic potential of the stem cells. These colonies were counted on an Olympus BX47 microscope with a 4x lens using the macroscopic view.

Specimen preparation

Study 1: 8 weeks after surgery, the animals were euthanised according to the approved animal licence by blood sampling and an overdose of ketamine and isoflurane. The implant bone blocks consisting of hydroxyapatite and newly formed bone were harvested and initially placed in 4% formaldehyde. After 24 hours, they were changed to PBS. The samples were then embedded in paraffin.

Study 2: After 12 weeks of observation, femur condyles were harvested and stored in a -20°C freezer for 24 hours to ease the removal of soft tissue without damaging the implant or new bone. The distal femur was then sectioned using an Exakt 312 diamond bond saw (Histolab Technologies,

Inc.) in a medial and lateral portion. The top washer was removed to achieve the correct angle to separate the inserted implant and gap in 1/3 and 2/3 sections (Figure 14, C). The 1/3 section was stored in a -20°C degree freezer for tissue preservation and mechanical testing (MTS), while the 2/3 section of the implant was methyl methacrylate (MMA) embedded.

Micro-CT scan

Micro-CT scans were attempted in the normal sheep; however, due to the technical settings of distinguishing between the HA substitute and newly developed bone formation, we were unable to include the scans. Thus, these results are not illustrated in this thesis.

The implant bone blocks were illustrated and analysed by a high-resolution µCT 50 (Scanco Medical AG, Brüttisellen, Switzerland). The scans included a 3D microstructural analyses of bone content including new bone and HA as well as a 3D reconstruction for visualisation. Due that the analyses would include both HA and new bone into the total bone volume, and not being able to distinguish the bone and substitute, the values could not be compared to those of allograft.

Notably, microstructural analyses are usually able to measure the new bone volume fraction between the implant and the host bone, which is defined in this design as the gap and was the volume of interest (VOI). The 3D construction could serve as an indication for the placement of the bone onto the implant and for the general 3D distribution of new bone within the gap.

Micro-CT scanning will be performed in future studies after the MMA embedding but before histomorphometric sectioning. All of the specimens will be scanned in the same orientation and the definition of VOI will be 12 mm from the centre in of the implant with 500 slices in all groups. The scanning program will have the following parameters: voltage of 90kV, current of 114 µA and a high integration time of 1500 ms to acquire optimal images. Each 3D image dataset will have a final voxel size of 5*5*5 μm^3 (5880x5880x1151 voxels).

Histology and histomorphometry

An evaluation of the structural component of the bone blocks was used for histomorphometric analyses. This is a valuable method for gaining knowledge on the bone remodelling process and the general containment and placement of generated tissue.

The theory of the embedding process is divided into several phases. The first phase is fixation and refers to the use of chemicals to preserve the natural tissue structure and prevent cell structure degradation (88). The next phase is decalcification to protect the cellular and fibrous elements of the bone from damage. In this step, the aim is to remove water from the selected tissues in order to solidify them and facilitate cutting. The final phase is embedding so the tissue can be sectioned and stained for evaluation (88) (Figure 11, T4–T5).

Study 1 consisted of the subcutaneous implant bone block, while Study 2 was performed with the remaining 2/3 implant and the other 1/3 was used for the mechanical test (Figure 11, T3 and Figure 14, D).

In Study 1, the entire implant was included in the analyses and defined as the ROI, with sectioning through all areas. In Study 2, there was an angle of approach that required randomisation. The implant was rotated around the axis of the titanium cylinder to define the angle, followed by frontal sectioning through the middle of the implant. This vertical section principle ensures that no preferable gap area was selected (89).

Study 1:

Paraffin embedding and preservation was initiated by the storage of bone blocks in 50% ethanol in a tissue cassette and dehydration with 10-minute interval in ethanol (50% increasing to 100%). The ethanol was then changed with a 2:1 ratio of xylene followed by 1:1, 1:2 and then 100% xylene at 15 min intervals. Finally, the samples were changed to a xylene-paraffin solution for final embedding.

Each implant was divided into four sections with 4µm in thickness and a separation of 100µm. The first three sections were stained with hematoxylin and eosin (HE) for the basic calculation of bone formation and the final section with human vimentin (hVim) to confirm the origin of MSCs from ovines (29,69).

Bone formation was evaluated by HE staining. The effect of the VEGF groups was compared to the control (MSCs). Predefined regions of interest (ROIs) were calculated by Cavalieri's principle using stereological software (newCAST, Visiopharm, Denmark). The predefined region in this

design consisted of all the tissue from each implant bone block at around 500–800 points for representable results (89,90). The tissue within the ROIs was classified as bone (B), fibrous tissue (Fb), granula (Gr), miscellaneous (Mi), muscle (M) or marrow (Ma) (69) (Figure 12).

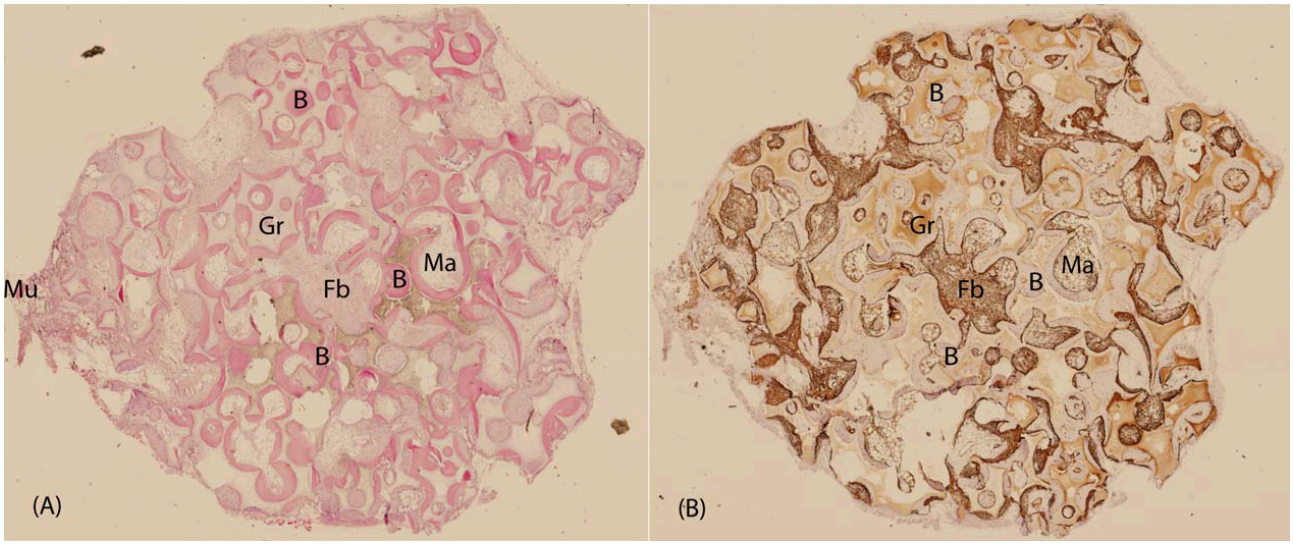


Figure 12: Sections stained with haematoxylin eosin (A) and human vimentin staining (B). B: Bone, Fb: Fibrous tissue, Gr: Granula, Mi: Miscellaneous, M: Muscle, Ma: Marrow.

Furthermore, additional sections in the middle of the implant were stained with HVIM (Thermo Scientific, clone SP20, cat.no RM-9120) to detect the origin of ovine cells due to the ability of HVIM to cross-react (29).

Study 2

The 2/3 bone block used for MMA embedding was fixated in different stages, with each stage lasting for 1 week. Each week, the tissue was dehydrated in ethanol (sequentially 70%, 80%, 86%, 99%) until being dehydrated with 99% ethanol + 1:1 acetone and a last step of dehydration in xylene. The fixation process involved 1 week of xylene + Technovit basic 1:1, while the following week involved Technovit basic + hardener, followed by Technovit basic + hardener + polymethyl methacrylate (PMMA) and a final embedding for 5 days according to department protocol.

After angle of randomisation was performed, the MMA-embedded implants were separated into four sections of 30um (Instrumentmarkerij, Medeja, The Netherlands; Figure 14, E) from bottom to centre for representative results from the entire implant.

The sections were stained with toluidine blue O for visualisation of new bone, fibrous tissue, granules and marrow. Each section was incorporated into the statistics by stereology. The new bone was verified by 2% light green and cover glass for clear visualisation of the lamellar structure within the area of new bone formation (89).

The stereological evaluation of the surface area of materials within the gap of the femoral gap model of these specimens has been verified in 2D by earlier studies. This has proven to be a reliable method in evaluating the vertical section design in relation to 3D results, thereby evaluating the entire gap in this model (91,92). The ROI was defined in two sections: zone 1 (close to the implant) and zone 2 (close to the existing bone), as described in the previous designs of the implant models (93–100) (Figure 13).

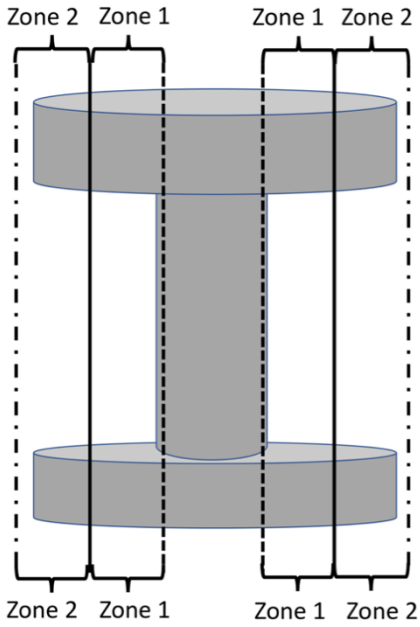


Figure 13: Illustration of zone 1 and zone 2 within the gap of the titanium implants

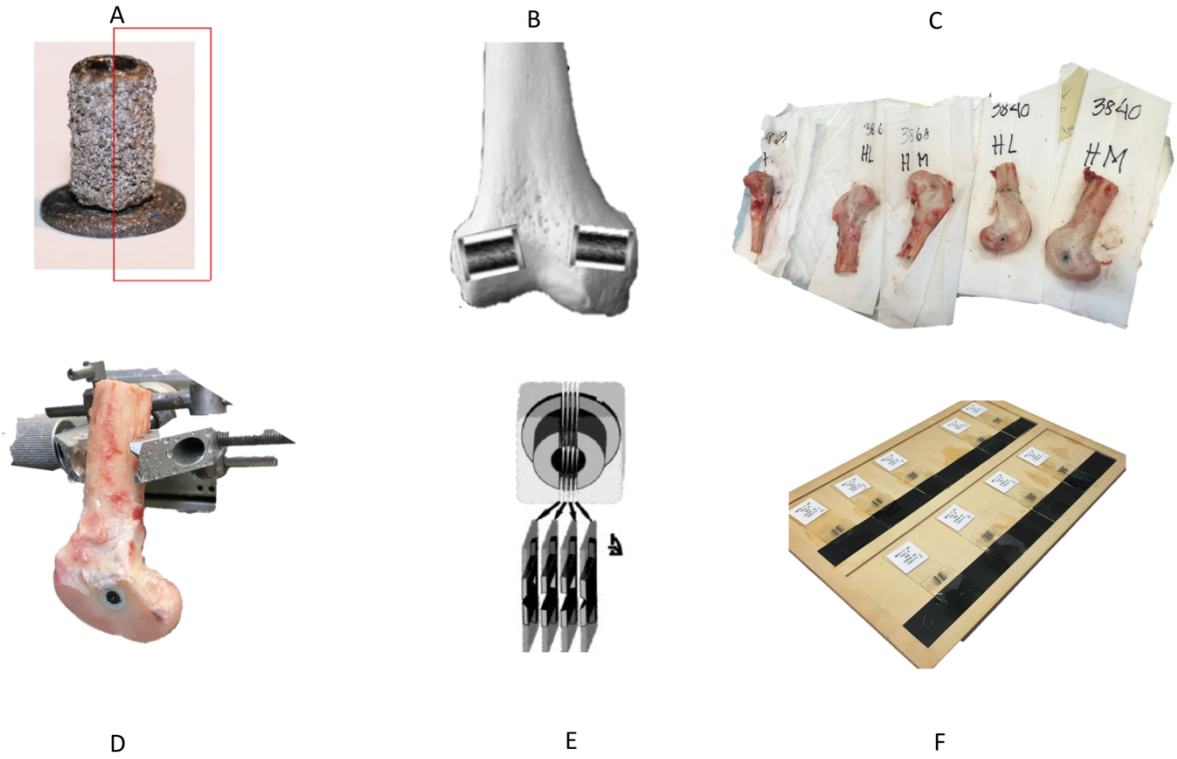


Figure 14: Illustration of the preparation procedure for the implants and tissue. A: Illustration of the Ti-6A1-T4 implant. The "gap" is indicated by the red marker. B: Position of the implant and materials in the trabecular bone in the medial and lateral distal femoral condyles. C: Distal femur divided into a medial and lateral portion to separate the inserted implants for individual sectioning. D: Implants are divided into 1/3 for mechanical test and 2/3 for embedding. E: Four sections from each implant prepared for histological evaluation after embedding was performed. F: Completed and stained implants ready for analysis.

Mechanical testing

Specimens were thawed at room temperature and testing was blinded for the researcher. All of the 1/3 bone blocks were tested in push-out to determine shear mechanical properties between the new bone generated in the gap and implant. The higher the load (N) that could be applied to the implant-bone interface before it failed, the better the bone formation within the implant and bone ingrowth into the porous titanium implant was presumed to be. The push-out test was performed using an 858 Bionix MTS hydraulic material testing system (MTS Systems Co, Minneapolis, MN) with 1kN cell load. The cylindric portion (piston) was applied to the implant in the bone implant block with ~3 newtons (N) for contact and position purposes. The piston has a diameter of 6mm and pushes out the implants with a rate of 5mm/min until failure. The load-displacement curve was recorded and converted to a stress-strain curve for calculation (101).

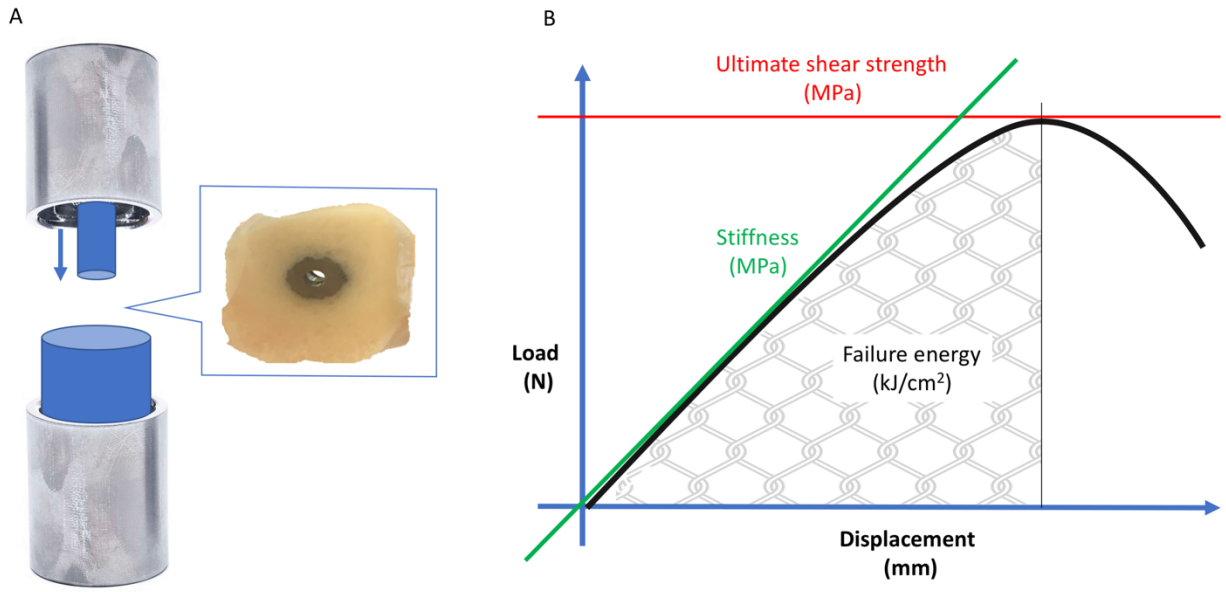


Figure 15: (A): Mechanical push out test. The area of force is the size of the titanium implant. (B): Stress-displacement curve with mechanical parameters

The ultimate shear strength that describes the first and highest peak and maximum stress before failure in the implant-bone interface in megapascals (MPa). Young's modulus (stiffness, MPa) is the maximum slope of the displacement curve and describes the rigidity and resistance to deformation of applied force. Failure energy (kJ/cm^2) is the area underneath the curve between zero strain and ultimate strain, i.e. the total energy absorption before failure (102).

Blood serum markers

All serum samples were distributed at -80°C and analysed at the Clinical Biochemical Department, Section for Pharmacology and Special Analysis, Glostrup, Rigshospitalet.

The chosen analyses were based on their ability to illustrate systemic bone formation in all models, focusing on osteoblasts, osteoclasts and osteocytes for the purpose of proving no systemic effect of our treatment to the host in order to establish a side effect profile for the dose response.

In the bone formation phase, both osteocalcin and pro-collagen type 1 (P1NP) are secreted and synthesised by osteoblasts. Osteocalcin exists in both bone and dentine, and has been strongly

correlated to general bone mineral density—especially in osteoporosis studies—as it is used as a preliminary biomarker in the medical treatment of osteoporosis (103). After being synthesised, P1NP is cleaved of the N- and C-terminal it utilises for collagen fibril deposition. The level of the C-terminal extension peptide (Pcoll-1-C) is a useful marker for the osteoblastic pro-collagen synthesis (104).

C-terminal telopeptides type I collagen (CTX) is a measure of the rate of bone turnover and a marker for the degradation by osteoclasts and their activity. High levels of CTX are thus correlated to an increase in the bone resorption phase. Clinically, high CTX levels will be inhibited by resorption-inhibiting therapy (105).

Osteoprotegerin (OPG) has a strong correlation with the receptor activator of nuclear factor-kappa B ligand (RANKL), also mentioned as the OPG/RANKL axis. OPG is secreted by osteoblasts, but also by other internal organs such as the heart, liver, kidney and B-cells. RANKL follows the activity of osteoclasts and is expressed in other tissues such as lymph, thymus and lung. The ratio between OPG and RANKL affects the axis, and with the high expression of OPG, the axis would be positive for bone formation and inhibitory for the RANKL expression (106).

Sclerostin is primarily produced by osteocytes and has an anti-anabolic effect on bone regeneration by inhibiting the Wnt signalling pathway (107), with the high expression of serum concentrations thus expressing decreased bone formation.

Serum markers: Study 1

During euthanisation at week 8, approximately 1ml of blood was collected using the retroorbital technique (Figure 10, day 64). The animal was fully sedated by ketamine/Rompun and isoflurane (as during primary surgery) and the procedure followed the protocol approved by the animal ethics committee.

The blood was then centrifuged for 10 min with 4000 relative centrifugal force (RCF) at 4ºC to produce ~500 ml of serum, which was isolated by pipette into 2 ml Eppendorf tubes and stored at -80ºC. The serum was then thawed and analysed for bone markers to detect the systemic activity of osteoblasts and osteoclasts (69).

Blood serum markers were measured at the Clinical Biochemical Department, Section of Pharmacology and Special Analysis, Glostrup, Denmark using the following kits and their individual instructions. Osteocalcin was measured using an ELISA Kit (Immutopics, Cat. #60–1305), pro-collagen type-1 (PINP) with EIA Kit (Immunodiagnosticsystems, Cat. #AC-33F), carboxy-terminal collagen crosslink (CTX-I) with EIA Kit (Immunodiagnosticsystems, Cat. #AC-06F1), osteoprotegerin (OPG) with ELISA Kit (Biomedica, Cat. #BI-20403), soluble receptor activator and nuclear factor – jB ligand (sRANKL) with ELISA Kit (Biomedica, Cat. #BI-20462) and sclerostin with ELISA Kit (Alpco, Cat. #41-SCLMS-E01) (69).

Serum markers: Study 2

Blood samples were conducted at different time points for the development of systemic markers. Baseline values was acquired and determined before the primary surgery, and measurements were then taken at weeks 1, 4, 8 and at euthanisation at 12 weeks. A total of 20 ml blood was collected from each sheep at each time point in two tubes, which were then centrifuged for 10 min with 4000 RCF for a total of 8–10 ml of serum that was stored in 5x2ml Eppendorf tubes at -80^{o}C.

The sheep focused on the systematic osteogenic markers in correlation to Study 1. Furthermore, we tried to illustrate the systemic level of VEGF with the local release during the 12 weeks of observation.

Blood serum markers were measured at Clinical Biochemical Department, Section of Pharmacology and Special Analysis, Glostrup, Denmark by following kits using their individual instruction. Osteoprotegerin (OPG) Sheep OPG ELISA kit. (Cat no: MBS2506141), receptor activator and nuclear factor – jB ligand (RANKL) Sheep Receptor activator of Nuclear Factor KB Ligand (RANKL) ELISA kit, pro-collagen type-1 (PINP) Sheep Pro-collagen Type I N-Terminal Propeptide (PINP) ELISA kit, Sclerostin, Sheep Sclerostin (SOST) ELISA kit, Cat no: MBS033198, VEGF Sheep Vascular Endothelial Growth Factor. (Cat. No: MBS737944), fructosamine Sheep Fructosamine ELISA kit, Bioassay Technology Laboratory, (Cat. No. E0122Sh) and osteocalcin and carboxy-terminal collagen crosslink (CTX-I) by iSYS immuno-diagnostic-systems IDS.

Sample size and power calculation

The sample size estimation was calculated using the following formula:

$$\frac{2(Z_{\alpha} + Z_{1-\beta})^2 \times \sigma^2}{\Delta^2}$$

where α is the risk of error of the first kind and β is the risk of error of the second kind. MIREDIF (Δ) represents the false-negative result.

The error of the first type $Z_{\alpha}$was 1.96 for a confidence level of 95% due to a two-sided effect. The critical value for β, the error of the second type, $Z_{1-\beta}$ was 0.84 due to the selected power of 80% (108). The minimal relevant difference Δ was selected at 70% and the standard deviation (σ) to 50% based on previous studies from the Department.

These calculations assume that we calculate whether the intervention group is as good as the control. Notably, the superiority analysis would require more implants being included.

According to these assumptions, at least eight samples should be included in each group.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to calculate overall differences between groups in between and compared to the control of allograft since, in independent unpaired data, we want to correlate it to a control group. Multiple comparisons were performed using the Holm-Bonferroni test (as appropriate) for normal distributions and the Kruskal-Wallis test for non-normal distributions.

Serum markers in Study 2 were calculated by matched one-way ANOVA since they are based on the individual at the same time, with multiple comparisons using the Kruskal-Wallis test. Furthermore, control of the two-tailed paired t-test was performed for control, and the direction of the serum values was not unidirectional.

P-values less than 5% were considered significant. The graphs and statistics were measured and constructed in GraphPad Prism v. 7 (GraphPad Software, Inc.).

Main findings

In this thesis, the primary hypothesis is that the use of MSCs and VEGF could serve as a permanent replacement for allograft in clinical use.

The studies in this thesis focused on achieving the optimal potential of the combination method regarding dosages, release timing and biological use with titanium surfaces for optimal bone growth. The results indicate that the therapeutic window of the VEGF in combination with MSCs is quite large since different dosages exhibited the same bone properties in our evaluation methods as in allograft.

However, when searching the literature for the use of VEGF for bone growth alone, this window seems to be quite narrow and largely dependent on dosage. Current strategies for the single use of VEGF seem to be further standardised before clinical application.

Main findings Study 1

The study concluded that, in an ectopic mouse model, the combination of mesenchymal stem cells seeded on HA with vascular endothelial growth factor show significantly more bone growth when stimulated with 10ng/day the first 14–21 days after surgery.

Histology

Histology was analysed based on regular H&E staining. This process can illustrate the total amount of new bone and tissue within the ROI, which consisted of the entire implant bone block of hard tissue extracted at euthanisation in this design. Furthermore, a human vimentin stain was performed to illustrate that the origin of induced MSCs from the sheep model was the cause of new bone development.

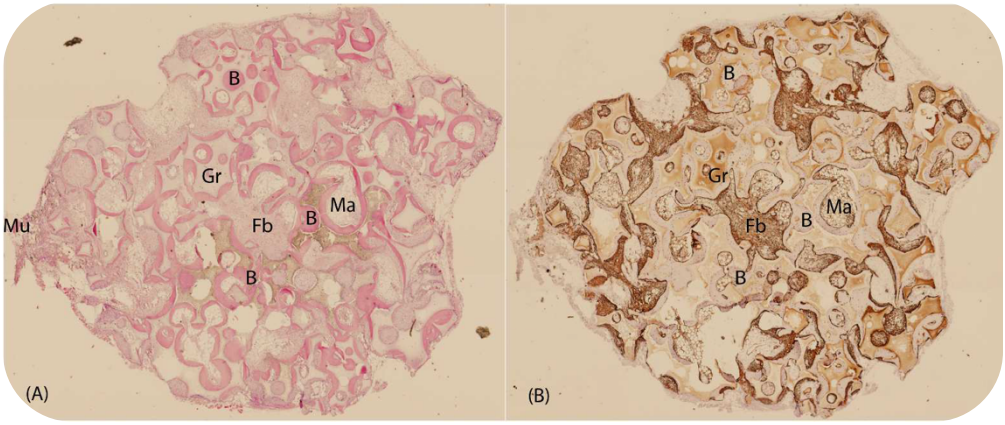
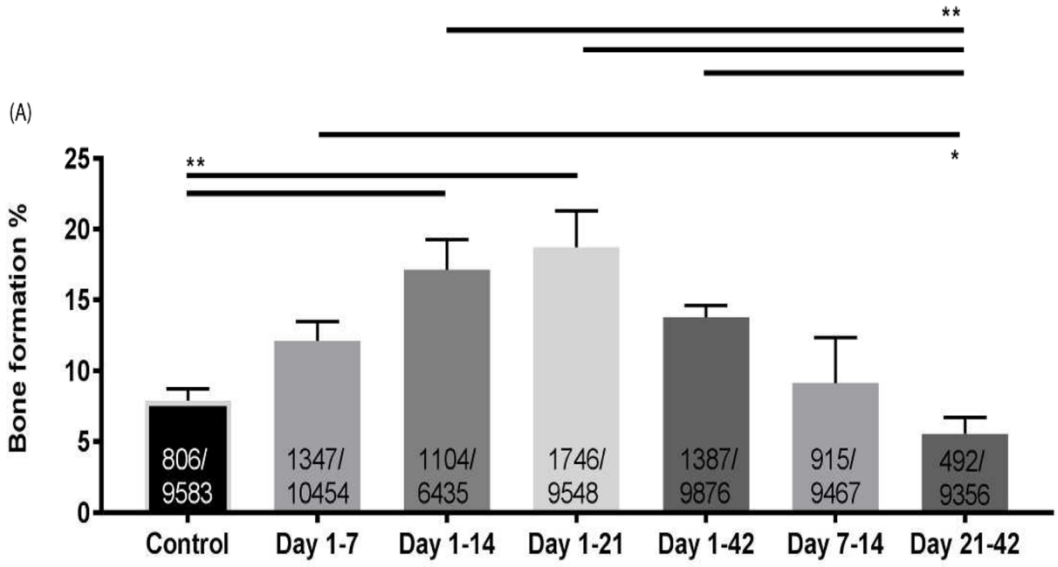


Figure 16: Histological images with (A) H&E staining in group days 1–14. (B) hVim staining from the same implant. The distance between section (A) and (B) is 100 lm. B, bone; Fb, fibrous tissue; Ma, marrow; Gr, granula; Mu, muscle. The hVim positive areas are brown indicating that bone originated from the cultured cells. These overview pictures were taken with newCAST, Visiopharm, Denmark, lens 34

The amount of new bone from each group on days 1–14 and 1–21 was significantly better than the control (MSCs only).



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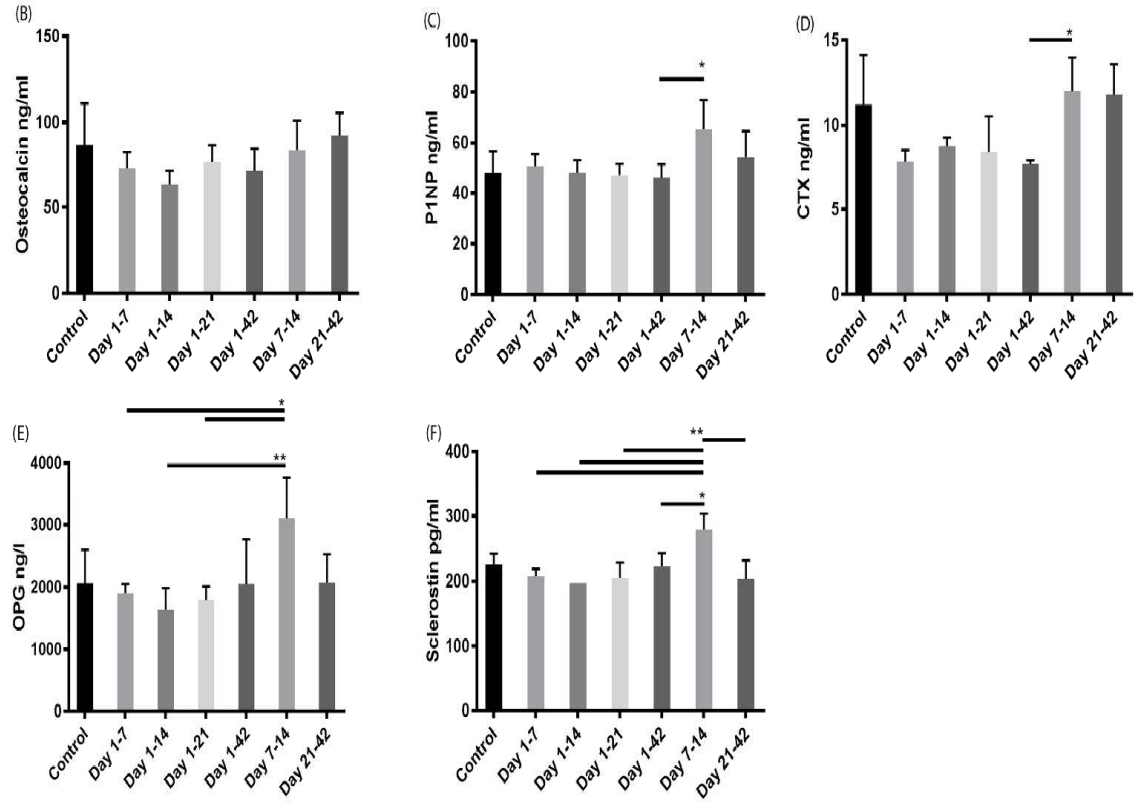

*Figure 17: A): Illustration of the bone formation (B/TV) in %. x/y; x amount of points for the counted new bone (B)/y the total amount of counted points from the entire group (TV). *p<0.05; **p<0.01. B) Osteocalcin, (C) pro-collagen type-1, (D) carboxy-terminal collagen crosslink, (E) osteoprotegerin, and (F) sclerostin. *p<0.05; **p<0.01*

Blood serum markers

For the systematic influence of induced MSC and VEGF, serum samples were obtained at euthanisation and analysed for bone-relevant markers. Notably, no marker had any significant difference compared to control (Figure 17, B).

Main findings Study 2

This study demonstrated that the combination of 3 x 10^6 million MSCs with a range of 10–500ng VEGF release for 21 days in a femoral implant gap model in normal sheep had the same bone-forming capabilities as allograft evaluated by histology and mechanical testing, with no systemic effect illustrated in serum samples.

Histology

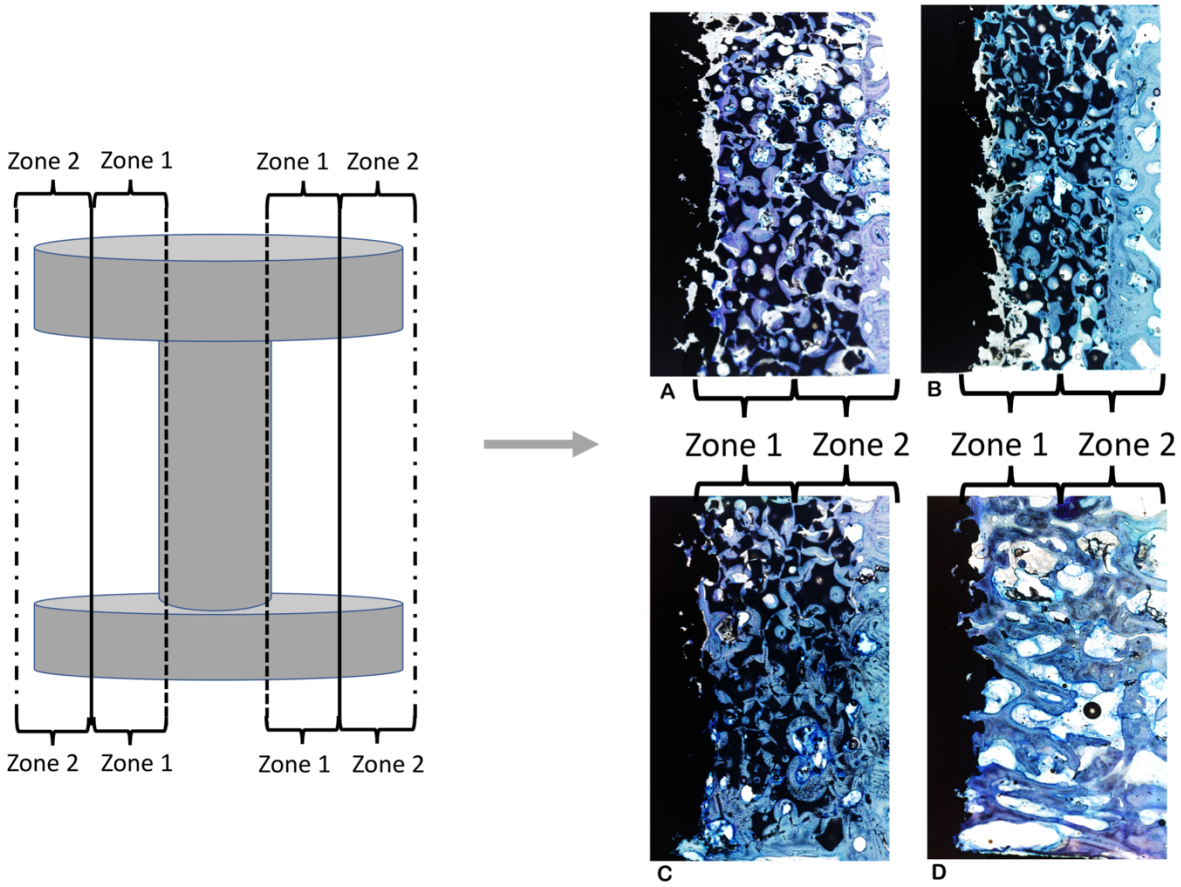

Figure 18: Illustration of the implant divided in zone 1 close to the implant and zone 2 close to host bone. Histological images from the gap in each group, stained with Toluidine Blue O after 12 weeks. A: MSC+VEGF 10ng/day B: MSC+VEGF 100ng/day C: MSC+VEGF 500ng/day D: allograft. These overview pictures were taken with newCAST, Visiopharm, Denmark, lens 4

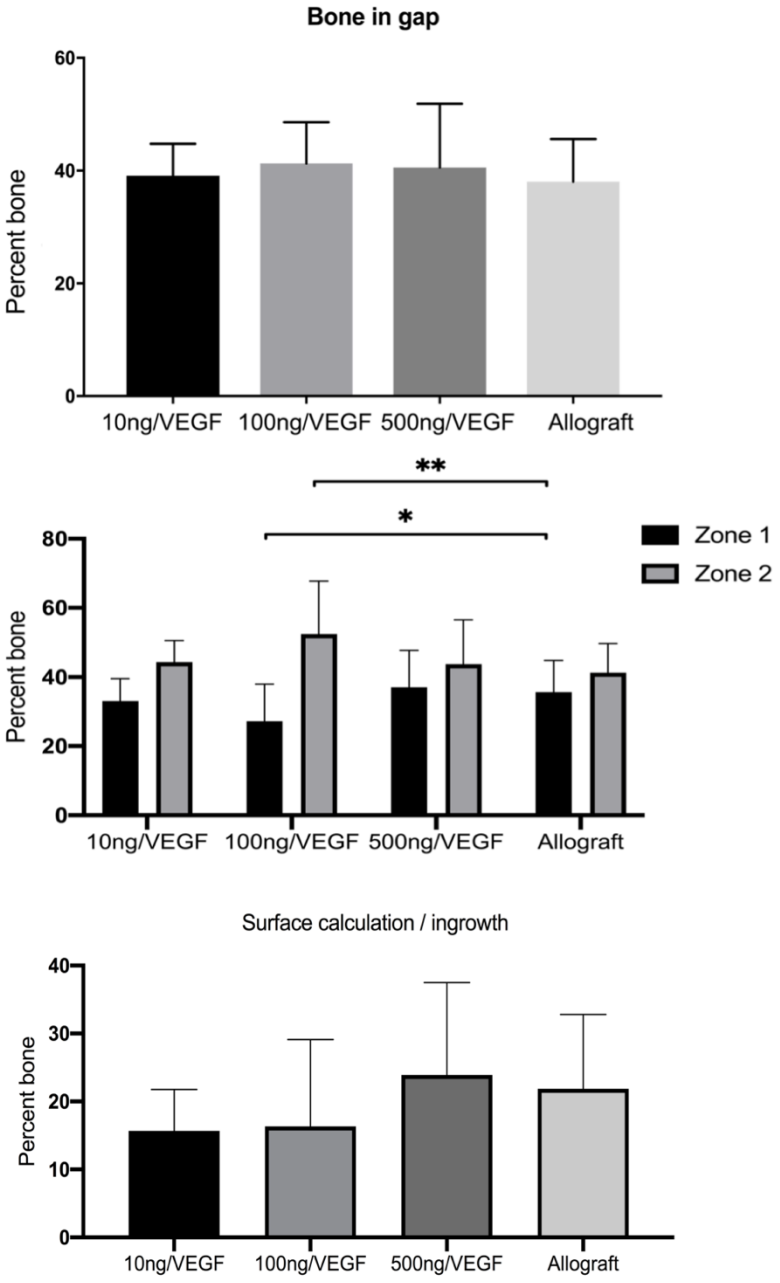

Figure 19: Percentage calculation of bone within the gap, from histological images. No statistical differences were seen between the groups in overall bone within the gap or in the bone ingrowth. 100ng/VEGF had lower percentage in zone 1 compared to allograft ($p<0.05$) and higher in zone 2 (** $p<0.001$).*

Mechanical testing

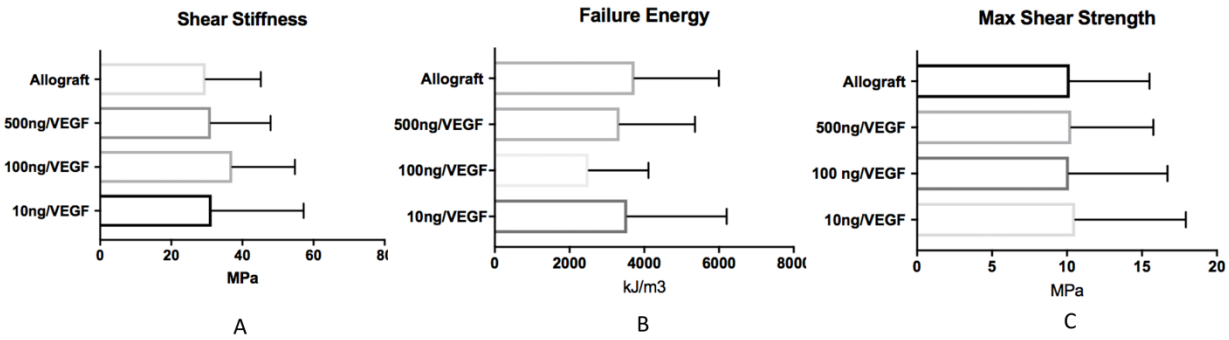

Figure 20: Mechanical testing from all groups defined by 3 parameters Shear Stiffness, Failure Energy, and Max Shear Strength. MPa (MegaPascal), kJ/cm^2 (Kilojoule/squate metre). No statistical differences were seen between each group in any of the parameters.

Serum markers

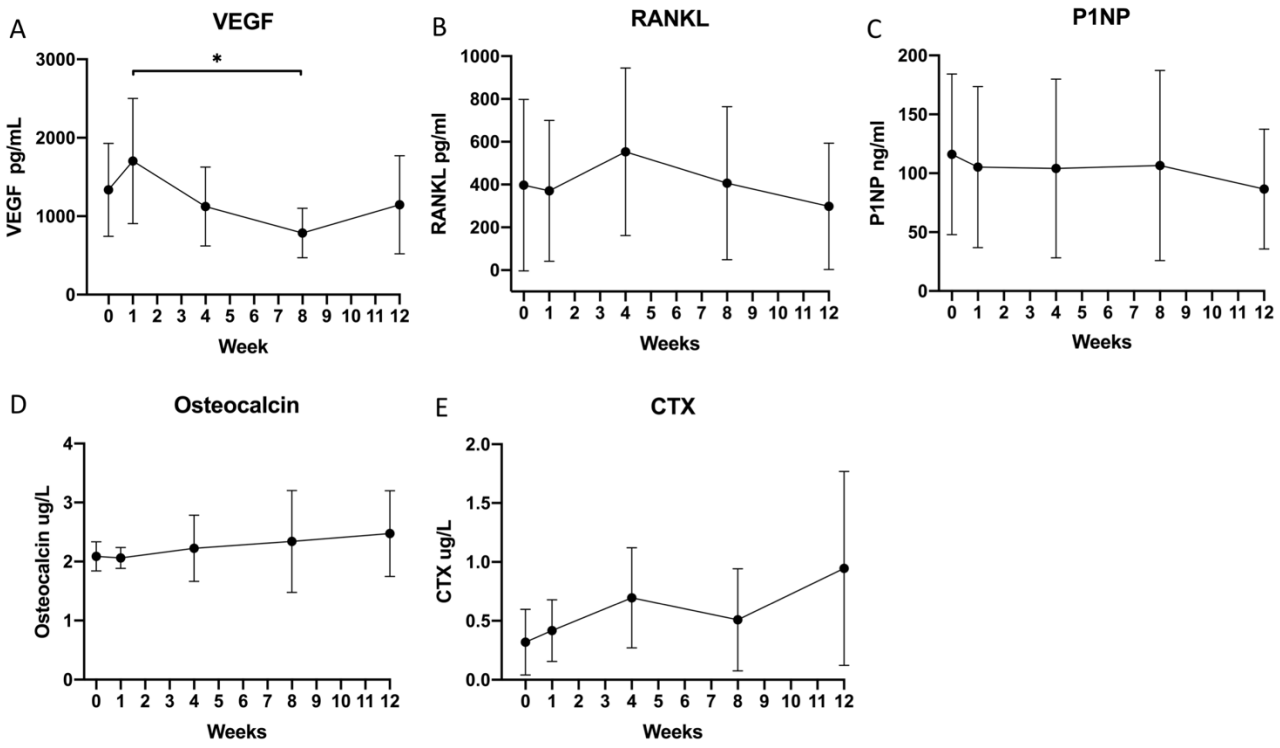

*Figure 21: Blood serum samples at different timepoints. A: Vascular endothelial growth factor (VEGF) B: receptor activator and nuclear factor – jB ligand (RANKL) C: pro-collagen type-1 (P1NP) D: Osteocalcin E: carboxy-terminal collagen crosslink (CTX). *p<0.05. OPG where below minimum value in all groups and time points. Sclerostin could not be measured by acquired kit in the sheep model.*

Main findings Study 3

This systematic review used reproducible search and inclusion methods to assess progress in the solitary use of VEGF for bone formation over the last 10 years, including the progress made towards clinical usage. A total of 1374 articles were found by the search criteria. Phase one included 70 articles by title and abstract, while and phase two excluded 46 by full-text analysis. Overall, a total of 24 articles met the criteria for inclusion. These were quality scored using eight validated questions and one modified for the purpose of this review. Notably, the use of many different models and methodologies made a statistical comparison difficult. However, some very exciting indications could be extracted from the articles, such as the most efficient use of VEGF appearing to be in defect models with the release of VEGF within the first three weeks, and in evaluation studies with an early focus of eight weeks or less appearing to have better use of VEGF. This review serves as an inspiration for future study designs in the modification and improvement of VEGF use for bone formation.

Search strategy

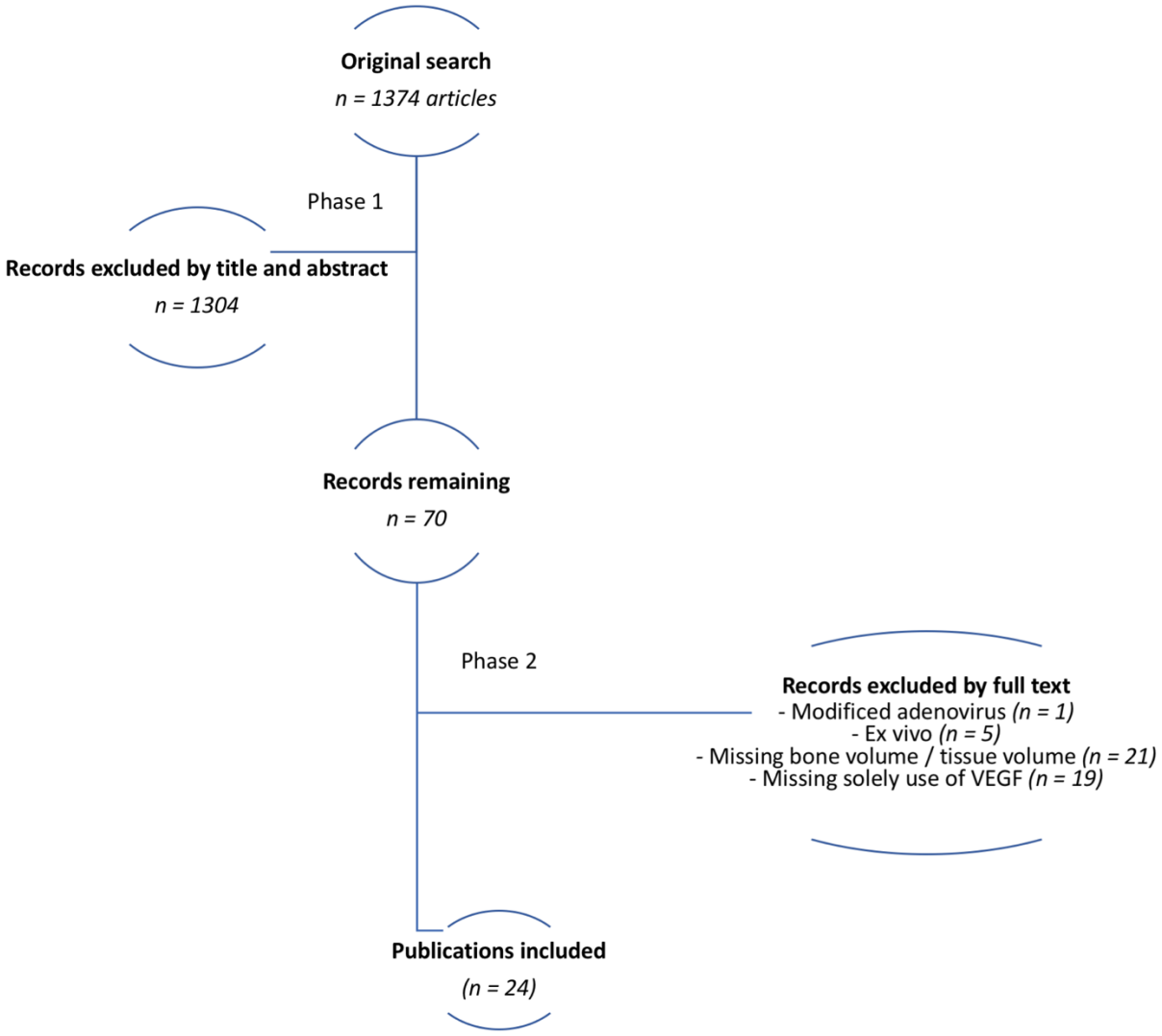

Figure 22: Illustration of the search strategy for the systematic review. A total of twenty-four articles were included.

Risk of bias / quality assessment

References	1) Peer review journal	2) Control group	3) Randomi-zation	4) VEGF dose justified	5) Blinding	6) Details on animal model	7) Sample size calculation	8) Comply with ethics	9) No conflict of interest	Quality Score
Amirian 2015 (23)	X	X	-	-	-	X	-	X	-	4
Kenney 2009 (32)	X	X	-	-	-	-	-	X	-	3
Lohse 2015 (24)	X	X	-	X	-	-	-	-	-	3
Çakir-Özkan 2017 (34)	X	X	-	-	-	X	-	X	-	4
L Zhang 2014 (42)	X	X	-	-	X	X	-	X	X	6
Lv 2015 (35)	X	X	X	-	-	-	-	X	-	4
Khojasteh 2017 (43)	X	X	-	-	X	-	-	X	-	4
Moser 2017 (25)	X	X	X	X	X	-	-	X	X	7
W Zhang 2014 (36)	X	X	-	-	-	-	-	X	-	3
W Zhang 2011 (37)	X	X	X	-	-	-	-	X	-	4
Quinlan 2015 (26)	X	X	-	X	X	X	-	X	X	7
Schliephake 2015 (27)	X	X	-	-	X	X	X	X	X	7
Behr 2012 (33)	X	X	-	X	-	X	-	X	X	6
Geuze 2012 (44)	X	X	X	X	-	X	X	X	X	8
Hernández 2012 (38)	X	X	-	-	-	X	-	X	-	4
Kempen 2009 (28)	X	X	-	X	-	X	-	X	-	4
Casap 2008 (39)	X	X	X	-	X	X	-	X	-	6
Patel 2008 (29)	X	X	-	X	X	X	-	X	-	6
Yang 2010 (40)	X	X	-	-	X	X	-	X	-	5
Yonamine 2010 (30)	X	X	-	-	-	-	-	X	-	3
Wu 2012 (41)	X	X	X	-	X	X	-	X	X	7
Schmitt 2013 (46)	X	X	-	X	-	X	-	X	-	5
Du 2015 (45)	X	X	X	-	X	X	-	X	X	7
Das 2016 (31)	X	X	-	X	-	X	X	X	-	5

Table 2: Modified score of quality by CAMARADES for systemic reviews in experimental animal studies (82)*: (1) peer reviewed journal; (2) control group; (3) randomization; (4)VEGF dose justified; (5) blinding; (6) details on animal model; (7) sample size calculation; (8) comply with ethics; and (9) no conflict of interests*

Strengths of these studies

Study 1: The methods used in this study are well defined and are well-known within the research unit through the handling and differentiation of MSCs for surgical procedures (29) and pellets having been used in more than 1700 international articles (IRA). The subcutaneous surgical design model has been described by one of the co-authors of this study (85), who also verified the suitability of reliable and blinded evaluation procedures (69). The purpose of the study is well defined, and the design can answer the hypothesis with separate groupings, with only one group within each mouse. Notably, two pilot studies were conducted to define certain variables such as the amount of MSCs in this design, mouse strain, evaluation time and VEGF release rate. The power calculation is sufficient with 12 implants per group for the histological evaluation.

Study 2: The femoral gap model is a well-validated model in Denmark and has been used for the past decade alongside MTS and histomorphometry for evaluation. MSCs were treated using the

same protocol in Studies 1 and 2, and seeded on the same type of HA; therefore, the results of the different models could be compared. The release of VEGF was correlated to the results from Study 1, while dosages were based on a pilot study on the amount of MSC for the best combination with a low dosage of VEGF. Blood samples were collected for any systemic effect of the localised stimulation. The control of allograft was produced and stored from protocol and matches previously obtained results on bone growth.

Study 3: This study was constructed as a systematic review for the transparent search- and evaluation of relevant methodology and was based on international standards for the scoring of animal studies. The review included 24 relevant articles that enhanced the possibility of a good representation of the hypothesis.

Limitations of the studies

Study 1: There were issues with the degradation of 2 pellets that were placed at the peripheral section of the implant. These were easy to define by histology; in the evaluation, the area of the pellet was excluded from the region of interest, thereby not affecting the statistics on other tissues. In the blood sampling, only a low amount of serum could be extracted, which was prioritised for the evaluation of osteogenic factors not including VEGF. Due to only four serum samples being available from each group, the strength of the statistics was weak; however, the values in each group are very similar. The only exception is OPG and sclerostin on days 1–14 which surprisingly were high in both groups, thus suggesting both an anti-anabolic and anabolic influence on bone formation. However, due to the divergent directions, this would not have changed the conclusion of the study.

Study 2: The control group for the largest impact in these studies should be autograft. However, this design was previously proven to have the same bone-forming capabilities as autograft in the sheep model, thereby serving as an optimal control in this study. The control of MSCs alone was only provided in the pilot study and could have been included in the primary study, even though results indicated the inferior effect of MSC alone. For greater statistical power, additional sheep could have been included, which could potentially have shown a difference between the groups. However, in this general gap design for a proof of concept, this amount serves the purpose of providing statistically significant results for future investigation.

Study 3: Notably, the search in this study only included PubMed, which could theoretically exclude some relevant articles. However, general search strategies were performed through PubMed and no other relevant studies have been presented in this thesis that were not included in the search string. Moreover, a statistical analysis was difficult to complete due to the diverse models and methodologies, which resulted in a lack of homogeneity. However, indications and patterns could still be made due to the thorough analysis of each article.

Discussion

The field of regenerative medicine has received a great deal of interest in recent decades. Notably, costs in the health care system are increasing as more elderly demand additional sources from hospital departments. Therefore, advancements in current procedures are becoming more essential than ever before. In bone research, many strategies are currently being investigated for optimal bone regeneration, with the further development of techniques and understanding regarding bone regeneration mechanisms being of great importance. Therefore, in this thesis, we focused on the methodology and outcome solely on bone regeneration.

First, we tested the optimal dosages and release rates of MSCs and VEGF in a small animal model in Study 1, which concluded that the best effect of VEGF occurred within the first three weeks when combined with MSCs. Then, study 2 focused on translational research through a large animal model on titanium implants to simulate the biological effect. Study 2 showed that, when using a combination of MSCs and VEGF in a large animal model, we can achieve the same amount of new bone as an allograft. This method is being investigated further through a future study in an osteoporotic sheep model with the same design compared to the use of VEGF only since Study 3—based on a thorough literature search—had divergent results regarding the use of only VEGF for bone regeneration.

Role of mesenchymal stem cells in bone research

Stem cell therapy has been developed and used in clinical studies for the past two decades (109,110). The literature views this as a crucial step for optimal healing, regardless of the general condition (7). The harvesting of these cells is also known to differ based on where they are harvested from (e.g. adipose tissue, skeletal tissue or bone marrow), while limitations such as age and comorbidities should be considered in application models (111). Within the medical field, other organs and clinical perspectives have found ways to fully utilise stem cells; however, it has not yet been applied as a clinical standard in bone research (112). Different fracture types have been investigated by randomised clinical trials (RCTs) from long bone non-union, to regular fracture models to arthrosis (110). However, these studies do not provide a clear conclusion regarding the effect on this intervention. For example, a review by Oryan et al. mentions that it could be due to inconsistency, a lack of expected results, or simply the need for further phase evaluation (110).

Approaches to the delivery of MSCs suffer the same problems as growth factors, while the primary methods have been to use injectable cells or seed them onto scaffolds. The next step has involved the co-delivery of cells and growth factors in an attempt to support the osteogenic, osteoinductive and osteoconductive properties of an optimal carrier. Regarding the cell, it may be favourable to support these with growth factors to improve colonisation and cellular attachment capabilities (113). However, due to the current lack of understanding regarding the exact mechanism and control of the differentiation process of MSCs, as well as the relevant safety precautions, the development of standardised protocols remains unavailable (110,114).

Using stem cells in the clinic

Stem cells have the same practical issues as autograft and allograft in that they must be harvested and stored correctly until use, and, in some cases, they should even be prepared in a lab and differentiated in the correct passages before being applied to patients. In Odense alone, different groups are working on solutions to these problems by using fresh stem cells harvested from adipose tissue (e.g. using stromal vascular fraction (SVF)) that can be prepared and used during procedures. Meanwhile, others focus on extracting cells and factors from peripheral blood and injecting them back into the patient in a localised area.

It should also be considered that the immunogenic response could produce a graft versus host reaction if a patient does not receive their own cells and reject the foreign tissue. A solution to this could be to modify the cells for personal markers or to medically prevent the immunoreaction. Some have suggested that embryonic stem cells do not cause the same kind of immune response (115). However, in elective surgeries, the viability of differentiated stem cells would still have a place in clinical practice. Ultimately, this comes down to the effect versus final cost for the aspiration and preparation of bone marrow.

In our experience, there is a huge variation in the tissue we collect our stem cells from, especially for bone regeneration (29). This can be caused by many factors, such as the harvesting procedure or the handling of the cells. For example, bone marrow extracted from the spina iliaca posterior superior (SIPS) compared to the proximal femur has also been indicated to have a higher density of stem cells (116). However, the collection of stem cells from the femur can be performed together with other surgical procedures (e.g. total hip arthroplasty (THA) and do not require further invasive procedures.

Angiogenic stimulation and growth factors in bone research

Growth factors that affect bone formation by directly promoting the osteogenic lineage targeting the osteoblast (directly or indirectly) as bone morphogenetic protein (BMP), transforming growth factor beta (TGF-B), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and VEGF are known to stimulate bone growth (117). However, a grey area in this field of research has always been the surrounding environment, which includes vessels, temperature and pH. It is common knowledge that sufficient vessel contribution to a localised area is important for tissue growth to supply nutrients, dispose of waste products and perhaps stimulate MSC homing (118,119); notably, this is where VEGF has an influence (18). This growth factor has been extensively studied (59,120) regarding its role in bone repair *in vivo* (Study 3). However, it seems to be very difficult to use in the optimal setting. For example, studies have shown that too much VEGF causes an excess of osteoclasts (121) or increase vessel permeability (63) and that VEGF should be released with the correct timing for optimal effect (69).

Due to the theory and divergent results regarding the effect of VEGF on bone formation, and after more than 20 years of usage and no clinical studies, it remains unclear whether this growth factor still has potential. In this thesis, the results indicated that it could have an effect similar to allograft in combination with $3x10^6$ MSCs; however, the control (only MSCs) without VEGF was only tested in the pilot study with lack of HA in the gap. Since the MSCs are seeded only on the HA, these samples had a very low amount of new bone, thus it can be assumed that MSCs alone did not have the same capabilities in this design as the combination therapy; however, no statistics could be acquired.

The crosstalk of osteoblast from MSC and angiogenesis is of great importance to new bone. Notably, MSC and endothelial cell (EC) interaction studies have shown increased bone formation (122). Some studies have mentioned that combination treatment is necessary to achieve the bone-forming effect of VEGF (118,123), whereas others have shown that VEGF works both alone and in combination with MSC on bone formation (124). Furthermore, a systematic review summarised the potential of VEGF alone in bone research (44) and other articles can be added to this literature search from before 2009 (36,47,67,125). Furthermore, studies on the blockage of VEGF receptors or the VEGF pathway have shown decreased bone formation and non-union (36,59). Despite this potential, in its evaluation of the last 10 years of relevant research, Study 3 could not show any advancement in this field based on inconsistent results and delivery issues (126). However,

angiogenic stimulation is a focus, though using different methods than the external stimulation of VEGF (e.g. by modified RNA (127) or in the 3D scaffold design to ensure sufficient vascularisation (128)). Furthermore, some studies mention that the use of VEGF is outdated; although the literature shows its potential, no existing techniques have reached clinical trials to date.

Moreover, it should be considered that other growth factors are known to have an effect on EC, though VEGF is the most potent factor of angiogenic stimulation (129). These other factors could be FGF, tumour necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), angiopoietins (Ang) or PDGF that is linked more to maturation and neovessel stabilisation (126).

Release methods and coating

Due to the typically short half-life time of growth factors, some type of verified release is necessary (126). Many methods have been mentioned in the included study of Study 3: "Gelatine (130–133), Plasmid DNA (130), PDLLA (134), fibrin glue (135,136), PLGA microspheres (137,138), PDLLA/CO3 (139), silk scaffold (140), hydrogel (141), alginate microparticles (142), PLGA microparticles (132), DNA oligonucleotide (125) and collagen sponge (143)". In our *in vivo* studies, we used verified pellets for the ectopic design (Study 1) and coated implant necks in the defect design (Study 2). However, components should always be addressed to suit the method and biocompatibility (118).

The pellets from Innovative Research of America (IRA) are verified by the company to be capable of releasing a specific amount of VEGF product depending on the corrosion of the pellet and total amount of product added. These are specially designed for subcutaneous implantation. However, a mouse study evaluated the release rate of the pellets and verified a burst release of the product, with the total release being performed within the desired period (144). This should be considered since Study 3 indicated a possible difference between burst and slow release according to VEGF.

In the coating method, the release was measured by Bradford assay ELISA analysis for the total release duration. However, this method could not verify burst or controlled release, as no baseline values were measured. Instead, the primary focus was to be able to reproduce and follow the results of Study 1 in securing VEGF release within the first 2–3 weeks.

While the pellets solved the issue of release in the subcutaneous mouse design, this method cannot be applied to clinical purposes. The coating methodology has more translational potential to

different orthopaedic materials, especially since the titanium implants used in our study were produced by Zimmer Biomet, who also produces materials for clinical use. However, coatings require modifications to adapt to different locations (long bone, vertebrae, etc.) metal surface areas (K-wire, gamma nails, etc.) and defect types (trauma, arthroplasties, etc.). This can prove difficult in terms of obtaining approval for several clinical purposes, making it even more important to focus on a specific clinical challenge in research in order to make as few changes as possible in the transition. Optimally, the coating could be calculated as an amount of liquid and growth factor per area of metal. This would provide a ratio that could be translated between different areas within the same type of defect.

Hydroxyapatite

This well-known substitute comes in many compositions of Ca/P ratio or with other materials (e.g. ß-TCP, PDLLA or calcium-deficient hydroxyapatite (CDHA)) (83,145). Furthermore, it is produced in different shapes, from blocks to granules (Fin-Ceramica). It also comes in different pore sizes and the structure can be produced to mimic the dimensions and shapes of calcified tissue via nanotechnology (146). The HA content in bones is a balance between strength and the corrosion rate. As such, the material should be strong enough to withstand pressure, while also allowing new bone to be formed in the localised area (145).

Many advantages and disadvantages exist in the composition and production of this material (147). In this thesis, we used pure HA, which has previously been shown to bear no osteogenic properties in the femoral gap model. Furthermore, the same type of HA could be used in Study 1 and Study 2 without any changes to the content, structure or pore size. Notably, changing as few variables as possible in the study designs increased the translational potential of this thesis.

Autograft and allograft

Many relevant reviewers and researchers seem to disagree on whether the use of allograft or allograft should serve as the positive control in bone graft research. This discrepancy is based on one's preference for using "living" bone with living cellular elements and osteogenic and osteoinductive properties from autograft or "dead" allograft bone that have been stored at -80^{o} with only osteoconductive properties (24). In our specific design for the femoral gap model in normal sheep, we previously described no difference in the bone-forming capabilities of these two types of bone grafts (99), thus we are comfortable in using the allograft model as a control. In academia, we refer to the allograft as the gold standard, which is also the case in this thesis. However, the general

opinion among clinicians is that if you aim to prove the effect of an intervention, it should be compared to autograft to prove the superior effect of existing methods. As such, this point should be taken into consideration when developing devices for clinical testing (148).

Blood samples and side effects

General markers for serum bone formation have not changed in recent decades, and their purpose also extends to diagnostic purposes (149). The focus and reasoning behind the chosen samples in this thesis were the desire to use the most common samples that could represent both the formation and resorption phases of bone remodelling. In Study 2, sheep provides enough serum to also focus on the systemic progression of VEGF. However, this design can only illustrate development within a certain duration of time, from before surgery until termination. In order to comment on the level of the samples, an additional eight sheep should have received an empty implant in the same size defect without any MSCs or VEGF to serve as a control for the specific systematic levels. Our primary objective with this evaluation was to detect any systemic effect of the local stimulation. Compared to before surgery, all of our groups did not exhibit any significant change, except in VEGF from week 1 to week 8. However, this is expected due to the systemic rise of VEGF following bone defects.

During the study, animals were observed daily by an animal technician or researcher for any obvious signs of discomfort. In Study 1 and 2, our treatment had no side effects on bone formation markers or the general condition of animals.

Comparing results from mechanical properties, Zone 1 and surface ingrowth calculations

When evaluating new bone formation in orthopaedic research, it is important to comment on the distribution and especially the incorporation of the new bone onto the osteosynthetic material. Notably, we have different ways of evaluating the strength of the new bone and implant connection. For the physical test, we used mechanical testing to determine how much force an implant within the new bone can resist in terms of applied and increased pressure. Using histomorphometry, we divide the implant into two zones, where zone 1 illustrates the new bone around the implant. Also, a specific surface calculation on the surface of the implant is performed.

We observed a significantly lower bone volume in zone 1 in the 100ng/VEGF groups when compared to allograft. For the ingrowth surface calculation and in the MTS, no significant

differences were observed between the control and intervention groups (Figures 19 and 20). This can indicate an equal distribution of new bone in the intervention groups in both the surface contact towards the implant (zone 1) and existing bone (zone 2). In conclusion, the results acquired from histomorphometry fit the results from mechanical testing and support the importance of the results for the clinical setting.

However, for optimal mechanical testing with the best referral to the clinical scenario, force (N) should be applied in smaller quantities over a longer period of time instead of measuring the ultimate shear strength since this context would simulate a patient's continuous use and pressure on the applied materials. However, since we work with sample tissue, this is not feasible and must be considered as a difference between academic and clinical evaluation.

Mechanical push out test

Some of the disadvantages of this measure are its destructive nature. Ideally, three tests per implant would have given a more reliable result, as the results show some level of diversity. Furthermore, studies have shown an error margin of up to 15%, which highlights the need for a higher difference in our substitutes for a significant difference (150). From an R&D perspective, an implant would typically not be exposed to uniform destructive stress, but rather continuous multiple stress in different biomechanical directions. However, the mechanical test continues to serve as a good and recognised method for testing general strength and exhibits no significant difference to the control, while it represents a valuable measure of the full assessment and comparison in our design between implants.

The future testing of mechanical properties will likely be focused on the different stages of the failure process by *in situ* uCT scans during this process, which may facilitate a more explanatory evaluation of these implants.

Osteogenic focus with angiogenic stimulation

The VEGF protein is known to be the primary stimulator of vessels, as shown in many previous studies (151–156). Based on the current literature, it is common knowledge that vessel distribution is enhanced by this simulation. The primary objective of this thesis was to develop a method that can replace allograft in the orthopaedic setting in a proof of concept design using different models. This applies to the different methods in attempting to achieve and prepare for future applications in

the clinical setting. Primary evaluations are based on bone-forming capabilities within a gap or region of interest (ROI) as well as the markers that contributed to methodology and design. For a deeper understanding of the bone formation results, future designs could focus on vessel distribution to clarify why our method presented enhanced bone formation with the stimulation of VEGF to seeded MSCs. An obvious theory is that the distribution of vessels within a region or a defect will provide assessable access to nutrients, cells and growth factors within the area, which can stimulate the bone healing process. However, an angiogenic assessment is required in order to fully understand the results.

Strengths and limitations in this thesis

This thesis was constructed using well-known and validated methods and models previously used by the Department, while also using the novel contribution of combination treatment and coating to develop a usable methodology for future treatments in the field of orthopaedic surgery. All methodologies and dosages were tested with pilot studies before their initiation into a primary study. The hypothesis started in a small rodent animal model for the proof of concept and was then incorporated into larger animal models for better a comparison to human tissue.
However, the results of this thesis are based on experimental research in animals, which will not achieve perfect translation into human tissue regardless of the animal model. Therefore, our results only stand as an indication of the expected results when evolving into clinical trials.

While the models we chose are relatable to clinical settings, both the subcutaneous model and the trabecular femur defect model do not correlate directly into a specific clinical issue; instead, they serve as proof of concept for more focused research. For example, the subcutaneous model could be correlated to vertebroplasties in the spine since the regeneration is outside of a defect. However, it is still placed subcutaneously and not on the bone level beneath the fascia. It must also be assumed that if the bone formation capabilities are promising, it should be further enhanced when closer to larger vessels and more surrounding tissue.

For example, since the femur gap defect is localised in trabecular bone, it is difficult to use in larger trauma defects since this design cannot comment on cortical formation. However, using it in anterior cruciate ligament (ACL) reconstruction or in arthroplasties—where the generated bone is primarily in the trabecular bone—seems reasonable.

Conclusion

Study 1: This study demonstrated that, in an ectopic mice model treated with seeded mesenchymal stem cells on HA granules, bone formation can be significantly increased compared to only MSCs following stimulation with VEGF at days 1–14 and 1–21.

This is a very important perspective for the optimal effect of angiogenesis and is theoretically correlated with the inflammatory phase and callus formation. When adding VEGF in the remodelling phase after 3 weeks, it seems to have an inhibitory effect. However, groups without VEGF in days 1–7 had the worst bone formation, which could indicate the importance of the first week. Moreover, when only stimulating in days 1–7, we did not observe a significantly enhanced effect.

Study 2: This study represents a further development in the method used for Study 1. This demonstrated similar bone formation, ingrowth, and mechanical fixation in the MSC and VEGF combination as those of allograft in the femoral gap model in normal sheep after 12 weeks of observation.

This study was based on the current knowledge from Study 1, and we attempted to compare to the current standards in allograft. Our results suggest that the methodology has potential in this combination for future designs in defect models.

Study 3: This study was a systematic review that aimed to present developments toward reaching clinical applications over the last 10 years. While no further progress towards clinical practice was observed, the trends of the large inclusion group illustrated some promising indications for future designs. When using VEGF for bone formation, it seems to have an optimal effect on bone defects, suggesting that the release should occur within the first 3 weeks and that early evaluation before 8 weeks presents better results compared to control.

While this thesis does not contain the solution to bone defect remodelling, it provides some predictions regarding the type of research that should be focused on. It also gives the opportunity to create a larger scale design with a specific focus on clinical trials.

Furthermore, it has provided a strong indication of the need for angiogenic stimulation in optimal bone growth. The results of this thesis can also lead to future designs wherein we can initiate the single use of VEGF compared to combination treatment in an osteoporotic sheep model.

Perspectives

Future translational medicine

When working in basic science, the intentions should always be on clinical outcomes and how to confirm/deny our research focus. This means focusing the research question to a clinical situation but comparing it to current or upcoming clinical standards. The natural progression is to test the hypothesis *in vitro* and consider the 3 Rs when performing *in vivo* studies. However, the problem is that many *in vitro* results do not show the same results *in vivo*, while even *in vivo* results can differentiate between different animals, species and locations. The solution to this problem involves global collaboration and not keeping the reproducibility potential within each research unit. Study 3 is the perfect example of a large body of *in vivo* research where the articles primarily focus on the novel contribution of a release method, with some not even mentioning the dosages of the VEGF product. The information presented in many of these articles are lacking and thus cannot be reproduced. This means that instead of evolving based on existing research, each team must start over with each new project. Therefore, there is a growing need for the standardisation of protocols and descriptions in this field and a reduced focus on making 'lucky guesses' according to methodology.

Furthermore, can our methods be used in a clinical setting? The best designs are made in communication with specialists or companies within the field. They know what is needed, which patients or bone structures would be relevant for inclusion, what can be implemented at the department and what is relevant in the design phase. Relevant questions include: which control group will have the highest impact for clinicians? Which control will convince clinicians/companies that this invention really works?

This crucial step should not be overlooked when focusing on funding and development for relevant issues. The same concern is applicable to practical matters. Can the device be sterilised? Can it be stored? Are they able to be approved by the FDA? Are they actually better than the current methods on the market? And if there is a current method on the market, do we use this as a control in our research and not just an empty gap? The final consideration is the cost-benefit analysis. If the

method is too expensive compared to the current, it will not reach clinical practice.

Fortunately, there has been more focus on these aspects in recent years, especially regarding the use of animals for research purposes according to collaborative strategies spanning continents and specialities, which will hopefully have an impact on developing this field and producing more quality research.

Future studies

Enhance bone regeneration and implant fixation in osteoporotic sheep

Introduction

Osteoporosis is a confirmed age-related disease that is growing in prevalence and thus defined as a current and future public health problem due to the ageing global population. This trend is expected to result in an increased treatment cost of 20–30% globally by 2030 (157). Osteoporosis is defined as a bone disease with the pronounced reduction of bone mineral density, causing the OC to resorb more bone than OB can produce, resulting in an imbalance between bone resorption and formation in the bone remodelling unit (BMU) that leads to bone loss (158). This states the importance of using verified models and testing in both normal and fragile bone structures for the best correlation to enhance the treatment for a relevant clinical situation. This study is designed and based on results from this thesis, where we tested the best combination of MSCs and VEGF from Study 2 and then compared these to using VEGF only on implants and HA. The study was performed using the same surgical procedure as the femoral gap model, but in osteoporotic induced sheep. The hypothesis of this study is that the combination of MSCs and VEGF or VEGF alone can have the same bone-forming properties in osteoporotic bone relative to allograft.

Study in osteoporotic sheep

This study used the same operational design and evaluation procedures as in Study 2, but in sheep with induced osteoporosis. In total, 12 Texel/Gotland sheep were included. The sheep were ovariectomised (OVX) and injected with glucocorticoids (GC) i.m. five times per week for 7 months before surgery. During this period they received a calcium-deficient diet and a natrium block (ViloRock salt 10kg, Vilofarm A/S, Denmark) for water retention, as per existing protocols (10). The design focused on comparing the best results from normal bone into an osteoporotic bone structure and testing whether the VEGF coating on HA or implants without any MSC would have the same bone-forming qualities as allograft and HA or implants in combination with MSC.

No significant differences were observed between the groups from Study 2 in normal bone, though there was a tendency in the use of 100ng/VEGF to not have as much bone in zone 1 (close to the

implant), while the use of 500ng/VEGF had a higher bone-forming percentage. Therefore, the most promising group was 3x10^6 MSCs combined with 500ng VEGF release/day.

	MSC	VEGF on HA	VEGF on implant	Only HA in gap
Group 1	3 x 10^6	-	500ng release/day	-
Group 2	-	500ng release/day	-	X
Group 3	-	500ng release/day	500ng release/day	-
Group 4 Allograft	-	-	-	-

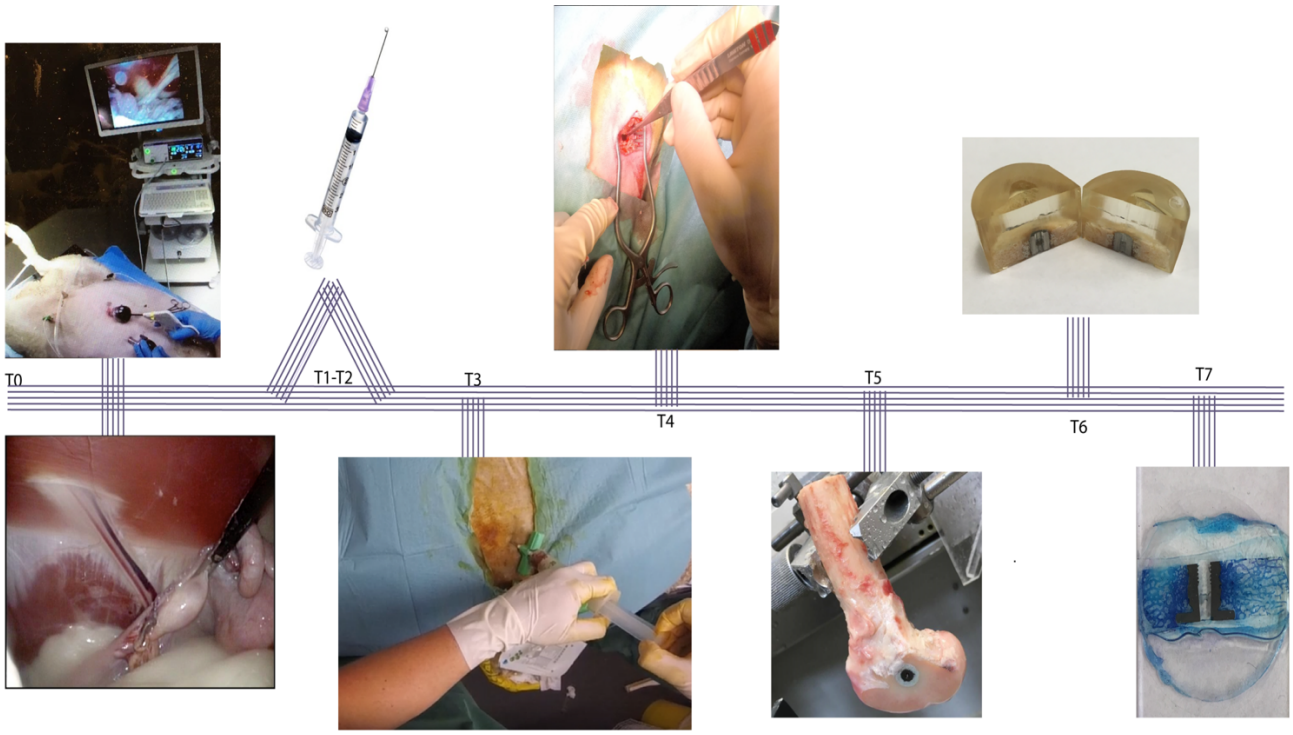

Figure 23: Illustration of the order of intervention in current OP study: T0 (week 0) Laparoscopic ovariectomy T2 (week 1): Glucocorticoids injection + Ca-deficient diet. T3 (week 25): Aspiration of bone marrow for cultivation of MSC. T4 (week 29): Surgery femoral implant gap model. T5 (week 41): preparation of samples for MTS and embedding. T6 (week 53) samples embedded and ready for sectioning. T7 (week 54) samples stained for calculation.

Osteoporosis induction

Ovariectomy: Sedation was provided according to the femoral gap model protocol with Rompun, propofol, isoflurane and Temgesic. The abdomen was disinfected and five markings of the entrance were made with red light visualisation and consideration for larger vessels. The cavity was filled with CO_2 using a pneumatic insufflation needle, and three trochars were placed. Using slide lock graspers, the ovary was located in the cavity and electrosurgically removed. The procedure was performed on both sides, resulting in the removal of both ovaries. Entrances were closed with single suture and sprayed with wound plast (Figure 23, T0).

Prednisolone: Each sheep received 0.1 mL /kg dexamethasone i.m. for 5 days per week for 6 months. The current weight of sheep was updated each month and dosages were correlated to their development. In the final month, there was a gradual weekly reduction of 25% of the dosage for a total of 7 months of steroid injection. In the final week before surgery, sheep did not receive any injections. Following surgery, the sheep did not receive any prednisolone since former studies have proven no rebound effect in the trabecular bone within this observation period (10).

Evaluations will be based on the same methodology of Study 2 with stained sections, mechanical testing and serum sample biomarkers. Over the duration of Study 2, the micro-CT program for differentiation between HA and new bone were developed and compared to histological results for representative results. Blood serum markers shared the same time points and assays as in Study 2, which facilitates the comparison of results between the two studies. Furthermore, this study contained bone-related markers of osteoporosis and blood glucose to both confirm the osteoporotic status and to monitor the distribution of glucose due to studies suggesting that diabetes induced by steroid treatment can influence bone growth (159,160)

Efficacy of tissue engineering in avascular environment

Introduction

More than 200 years after the first production of magnesium (Mg)-based material implants, their use in both cardiovascular and orthopaedic surgery is still being investigated (161). The main effect of Mg is its corrosion capabilities, though it also has various effects on different cell lines and angiogenic expression (162,163). In the field of orthopaedic surgery, the optimal use of Mg could result in a reduced need for second operations due to implant removal. This could be beneficial for both patients and global healthcare costs.

The major concern when working with degenerable Mg is the reaction of the host and surrounding environment. The corrosion and waste products are different depending on the precise containment of the material and generally consist of either pure Mg or Mg alloys. One of the main issues has been the increased degradation rate of Mg materials, which results in challenges related to organisms excreting these waste products. To delay this degradability, the use of coverage or combination with other materials has proven to be effective (164). In theory, using Mg/Calcium cement can decrease the surface area of the stent and can provide stability before corrosion.

Existing *in vitro* tests for Mg are divided into two broad categories: those concerned with the biocorrosion resistance/biodegradation behaviour or those concerned with toxicity/interaction with biological organisms (165). The effect on the surrounding environment and the corrosion rate of the implant is affected by pH value, making this a serious concern when using substances that can balance each other for a stable value (165).

In a saline environment, magnesium-based alloys will be degraded into magnesium chloride, oxide, sulphate or phosphate. It is believed that the release of magnesium ions from corroding magnesium alloys will not have any local or systemic toxicity in small amounts (166), but could actually have a positive effect on some cell lines (162). In hypoxic environments, Mg has shown no negative effect on angiogenesis (167). The concern is regarding the evolved hydrogen bubbles that can accumulate due to the corrosion of the Mg. These can turn into gas pockets that might cause the separation of tissue and metal (166). This suggests that the combination of Mg or surface coverage methods would be preferable (166).
Avascular necrosis (AVN) involves the progressive destruction of bone as a result of compromised bone vasculature, the death of osteocytes, and the deterioration of bone architecture. AVN can be a result of trauma, glucocorticoid use and alcohol abuse (168). If no preventative treatments are given, AVN patients with a large area of bone lesions (>30%) eventually suffer from hip joint collapse, which is also described as Association Research Circulation Osseous III (ARCOIII) (168). Once the hip joint begins to collapse, it will lead to the development of secondary osteoarthrosis (OA), which will result in most patients eventually requiring total hip replacements (THA) (169,170). In the US, AVN of the femoral head is one of the most common causes of hip disorders, with 20000–30000 newly diagnosed cases annually (171). Therefore, preventing the joint collapse and secondary OA of necrotic joints are substantially important for AVN patients to avoid expensive hip replacement surgeries. This study focused on the surgical treatment of stage II,

defined by association research circulation osseous (ARCO), for the prevention of the hip joint damage—thus avoiding total hip arthroplasty (THA). This stage is diagnosed by MRI via the lack of a crescent sign, which is a sign of collapse and the definition of stage III AVN (163,172).

The current gold standard for this preventative therapy of stage III AVN is core decompression (173); however, the practices in some places are moving towards the use of tantalum implants (172), with stem cells also being a focus in combination therapies for both the knee and hip (173,174). In the emu model, it has previously been shown that 100% of bone tissue in the proximal femur will be induced with necrotic tissue, while 70% will experience a collapse within months (175). When using core decompression as a control, the positioning and surgical procedure is very important for the treatment results, as the decompression should be very close to the area of possible collapse to have any effect. Our outcome for this study was hereby to develop a reproducible and translational operational technique in the emu model, where our objectives would be 1) to treat the general necrotic tissue within the bone and 2) prevent later collapse of the bone by using a specially developed magnesium stent alone and combined with Mg/calcium cement in a 1:1 ratio.

Method

The emu model has shown similarity in hip contact forces which makes it a biomechanically attractive animal for modelling general loading-dependent human orthopaedic disorders of the hip, and for the induction of avascular necrosis and subsequent collapse (175,176). The general design of the stent is made for the penetration of cement after insertion into the bone, with larger holes for penetration into the distal portion. The theory is to stabilise the fragile bone with mechanical strength and osteogenic properties (Figure 24). The cement is mixed with calcium to cover the surface area of the stent in order to ensure stability within the bone and to delay the magnesium corrosion. The total observation period was 12 weeks, as per the method article that defines the induction of steroid-associated osteonecrosis (SAON).

Animals

The emu is a large bird that weighs approximately 50 kg, is 1.5m tall and has a grown bone structure at approximately one year of age (177). Although it is not a commonly used animal model for bone research in general, it has shown verified collapse in the femur bone following the induction of avascular necrosis (175).

The emus were housed at the Shenzhen Emu Institute, Shenzhen under the general conditions of the farm. The animals had an indoor facility indoors and had and food available *ad libitum*.
This study included 30 emus with a total hip amount of 60.

Design

Magnesium stent

A pure magnesium stent was designed for the stent operation. It was placed at the lateral side of the femur just below the trochanter at an angle of 120 degrees, then through the neck and into the caput—preferably close to the induced bone collapse. The angle was guided by k-wires. The drills had diameters of 4.5mm and 8mm for stabilisation from the thread. After placement, bone cement could be injected through the screw and through the holes of the distal part to fixate the stent.

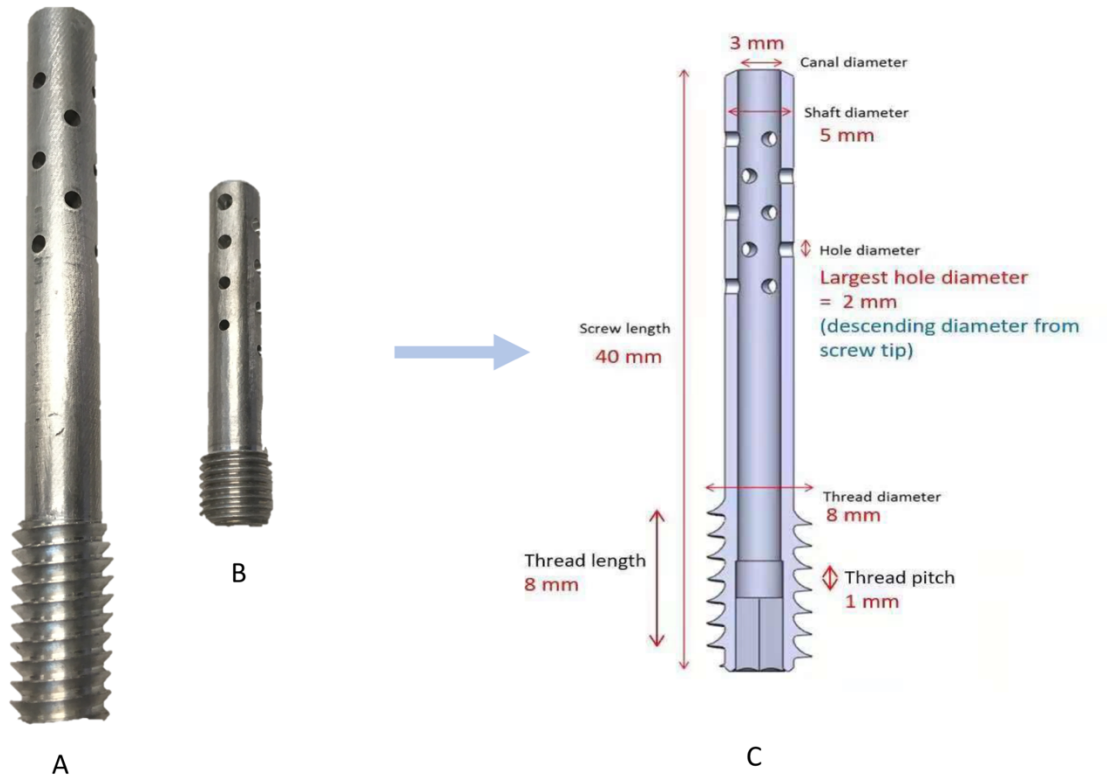

Figure 24: Measures for the Mg stent. A: Mg stent in human size compared to emu size (1:1 for R&D purposes). B: Emu stent. C: Design and size of emu stent with gaps in the distal portion for cement penetration.

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Cement

Mg anodes were constructed by the combination of Mg powder and calcium powder at a ratio of 1:1 as measured by the weight of each component. This was combined with a cement liquid of ultrapure water in the desired dosage. In *in vitro* studies, this solidified within 6–8 minutes after combination.

Design of study

Evaluation after 4 weeks. 15 emus

	Mg stent	**Mg/Ca cement**	**Core decompression**
Group 1	X	-	-
Group 2	-	X	-
Group 3	-	-	X

Evaluation after 12 weeks. 15 emus

	Mg stent	**Mg/Ca cement**	**Core decompression**
Group 1	X	-	-
Group 2	-	X	-
Group 3	-	-	X

Steroid associated osteonecrosis (SAON) induction in the emu model

The induction of SAON was performed for a total duration of 14 weeks. The emu would receive lipopolysaccharide (LPS) 8ug/kg at days 0 and 4 intravascularly (i.v.) in the jugular vein under full sedation with xylazine (2 mg/kg body weight). Furthermore, they would receive methylprednisolone (MPS) 10mg/kg i.m. at day 8, 10, and 12 in the thigh musculature. After 12 days, the induction period would be a total of 12 weeks before surgery. Due to previous complications with stomach acid, the emu were supplemented with 40mg of Omeprazole and 500mg x 3 of Amoxicillin orally for 7 days after induction (175).

Surgery emu stent model

After SAON induction by protocol (175), the emus were anaesthetised with xylazine (2 mg/kg body weight) combined with ketamine (50 mg/kg body weight) via i.m. injection. A feather cutter exposed the area of the proximal femur and the area was disinfected with ethanol (70%). The exposed leg was fixed with internal rotation to expose the great trochanter. A 5cm vertical incision was made lateral to the greater trochanter and soft tissue and muscle were split until the bone surface was reached. Two larger vessels in the area were fixed during surgery. A marker on the lateral plate was palpated and a K-wire was inserted at a 120-degree angle for 40 cm if no decrease in resistance was acknowledged by the surgeon (Figure 25, A). The placement was verified by a one-plate x-ray machine (Figure 25, D). A K-wire-guided sharp drill expanded the defect, first using a 4.5 mm diameter drill and then an 8mm drill (Figure 25, B). The distal 1cm of the tunnel was expanded by tapping. The residual bone fragments inside were removed and the stent was inserted. If relevant according to the randomisation of groups, 2ml of bone cement was injected through the stent followed by 8 minutes of hardening. The wound was then sutured in three layers and sprayed with liquid bandage.

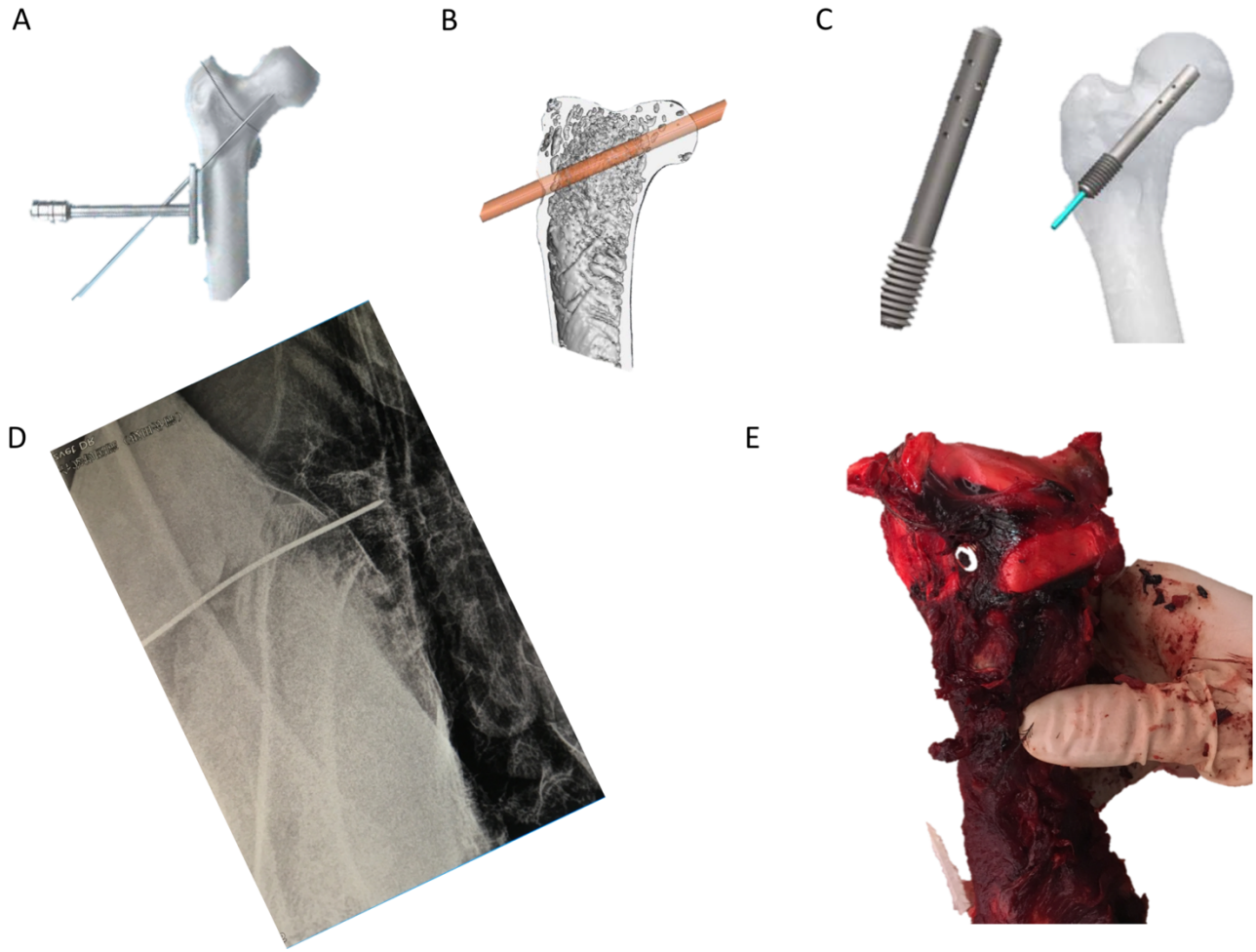

Figure 25: Surgical approach for the placement of the magnesium stent. A: Regular dynamic hip surgery (DHS) angle device for human use. B: 3D reconstruction of emu femur bone with the planned placement according to the size and length of manufactured Mg stent. C: Placement of the design for human use. D: X-ray representation of placement in emu bone by K-wire. E: Placement of stent design after euthanasia.

Evaluations

Evaluations focused on the effect on inhibition of the necrotic tissue from the inserted Mg material, which can be located by regular H&E staining. The density of the trabecular bone and collapse can be evaluated by micro-CT scan. Ongoing gait analysis was performed during the 12-week observation period to detect any clinical signs of collapse or discomfort.

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Appendices paper 1-3

Paper 1

Optimizing combination of vascular endothelial growth factor and mesenchymal stem cells on ectopic bone formation in SCID mice

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Abstract: *Introduction*: Insufficient blood supply may limit bone regeneration in bone defects. Vascular endothelial growth factor (VEGF) promotes angiogenesis by increasing endothelial migration. This outcome, however, could depend on time of application. Sheep mesenchymal stem cells (MSCs) in severe combined immunodeficient (SCID) mice were used in this study to evaluate optimal time points for VEGF stimulation to increase bone formation. *Methods*: Twenty-eight SCID (NOD.CB17-Prkdc^{scid}/J) mice had hydroxyapatite granules seeded with 5×10^5 MSCs inserted subcutaneous. Pellets released VEGF on days 1–7, days 1–14, days 1–21, days 1–42, days 7–14, and days 21–42. After 8 weeks, the implant-bone-blocks were harvested, paraffin embedded, sectioned, and stained with both hematoxylin and eosin (HE) and immunohistochemistry for human vimentin (hVim) staining. Blood samples were collected for determination of bone-related biomarkers in serum. *Results*: The groups with 5×10^5 MSCs and VEGF stimulation on days 1–14 and days 1–21 showed more bone formation when compared to the control group of 5×10^5 MSCs alone ($p < 0.01$). Serum biomarkers had no significant values. The hVim staining confirmed the ovine origin of the observed ectopic bone formation. *Conclusion*: Optimal bone formation of MSCs was reached when stimulating with VEGF during the first 14 or 21 days after surgery. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 105A: 3326–3332, 2017.

Key Words: mesenchymal stem cells, vascular endothelial growth factor, bone formation, severe combined immunodeficient mice, tissue engineering

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INTRODUCTION

Whether in relation to trauma, reconstructive, or alloplastic surgery, bone stimulation formation could prove essential in the field of orthopedic surgery. This type of formation could lead to faster recovery or fewer surgical complications in cases such as osteosarcomas, infection, or trauma which involve large quantities of bone or bone substitute to ensure sufficient bone repair.[1,2]

Combined with shorter hospitalization periods for patients, improvement of bone formation could result in better patient life quality and create a positive economic impact for the hospital.

Allograft is primarily used for large bone defects in clinics. Even though allograft has no osteogenic properties and carries no risks of disease transmission, bone banks often do not have enough[3] which emphasizes the need for a capable osteogenic biomaterial to meet the need requirements of patients.

In this study, pure hydroxyapatite (HA) was the carrier biomaterial used. HA is highly compatible with human bone[4] and is structurally ideal when compared to human trabecular bone which results in an osteoconductive scaffold which has optimal calcium-phosphate donor potential.[5]

Blood supply is the most common limitation for optimal bone formation,[1,6] and vascular endothelial growth factor (VEGF) is the main stimulator of blood vessels. This chemokine is derived from mesenchymal stem cells (MSCs) and endothelial cells which results in inducing angiogenesis by

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increasing endothelial proliferation and migration, vessel permeability, tube formation, and survival.[7]

The stimulation of VEGF[8–10] and MSCs combined with VEGF has been shown to enhance bone healing.[6,11,12] In spite of this, the method of delivery and optimal time of VEGF stimulation in bone formation has yet to be determined. A variety of methods for local administration of VEGF have been investigated: mixed with MSCs,[8] injecting into the area of interest,[10,11] or prolonging the release with hydrogel.[13]

The aim of this study was to investigate the most efficient time point for VEGF stimulation on ectopic bone formation. HA granules were seeded with MSCs and implanted subcutaneous (s.c.) in severe combined immunodeficient (SCID) mice along with VEGF-releasing pellets which had specific release profiles. These pellets are designed and distributed by Innovative Research of America (IRA; Sarasota, FL). Two pilot studies were performed prior to this study to evaluate the most suitable number of MSCs, mouse strain, time of observation, and release rate of VEGF for optimal ectopic bone formation in SCID mice.

Our hypothesis was that VEGF and MSC seeded HA would have an additive effect on the stimulation of bone formation in the early generative phase only when compared to MSCs.

MATERIALS AND METHODS

VEGF and pellets

The VEGF family has a half-life-time of between 4 and 24 h[14] which makes it difficult to induce VEGF during surgery for long-term effect without reaching the toxicity threshold. This study used customized pellets made of cholesterol, cellulose, lactose, phosphates, and stearates (IRA). These pellets have a biodegradable design and are specially constructed to contain and release the rVEGF165 at a certain rate in mice models.

Bovine serum albumin (BSA) is frequently used as a stabilizer for other dissolved proteins and binds water, salts, vitamins, fatty acids, and hormones as it carries these molecules between tissues and cells stabilizing the binding to the protein.[15] Accordingly, the pellets in this study contained rVEGF165 along with BSA in a 1:50 ratio (293-VE, R&D Systems) to enhance the binding of VEGF to the compound.

MSCs from bone marrow

Bone marrow was aspirated from sheep and transferred into a 50 mL falcon tube containing 4 mL of alpha minimum essential medium alpha (alphaMEMA, Life Technologies Europe BV, Denmark #22571–202) and 1 mL heparin. The tube was stored at +4°C.

The bone marrow was diluted with phosphate buffer saline (PBS, Dulbeccos, Life Technologies Europe BV, Denmark #14190094) at a 1:1 ratio; 13 mL Ficoll was also added. The resulting solution was centrifuged for 30 min. Between the Ficoll and the lymphocytes, the cell-containing middle layer was collected from all samples and mixed with 50 mL of PBS.

The cells were washed in MEM, centrifuged and resuspended in 10 mL of preheated mixture consisting of (37°C) alpha MEM, fetal bovine serum (FBS; Sigma-Aldrich, Denmark #F0804), and penicillin streptomycin glutamate (PSG; Life Technologies Europe BV, Denmark #10378016). One hundred microliters cell suspension was used for cell count.

The colony forming unit (CFU) cultivation was done for 14 days in an incubation chamber after which cells were washed with PBS and fixed with 4% formaldehyde and incubated for 5 min. After this, the formaldehyde was removed and the cells were washed twice with PBS. The cells were then incubated for 30–60 min and stained with crystal violet blue. The crystal violet blue was applied and aspirated and the cells were rinsed with water. Finally, the number of CFUs of the 1×10^5 MSCs was counted.

Subculturing of the cells was done at 80–90% confluence. Cells were washed with 10 mL PBS and detached from the culture surface using 3 mL of trypsin–ethylenediaminetetraacetic acid (EDTA, 0.05%; Life Technologies Europe BV, Denmark #25300–054) for 3 min. Seven milliliters of MEM was added and was followed by centrifugation of 200 *g* for 5 min, resuspension in 10 mL MEM, and manual cell counting. The cells were stored in tubes of 5×10^5 cells for further expansion or used for the preparation of implants.

One-milliliter syringes were cut open at the tip and placed in upright positions. They were then filled with 40 mg (±0.5 mg) HA, 100 μL growth medium, and cell suspension with 5×10^5 cells. The syringes were incubated for 15 min in 37°C 5% CO_2 after which the mixture was combined with VEGF pellets.

CFU. After cell isolation and before culture expansion, 0.5 mL cell suspension was subject to CFU. Cells (1×10^5) were used for CFU-cultivation and were calculated according to former protocols (Fig. 2).[16] The colonies were counted manually with an Olympus BX47, Tokyo, Japan microscope with lens ×4.

Substitute

The graft material consisted of HA granules with a diameter of 1.0–2.5 mm (ENGIPORE, Fin-Ceramica, Faenza, Italy) and was characterized by very high porosity which could reach 90% relative to the total volume (TV).

Animals

Mice. Twenty-eight NOD-scid SCID mice from The Jackson Laboratory (NOD.CB17-Prkdc^{scid}/J) were used. The mice in both pilot and primary studies were 8 weeks old with an average weight of 22.08 ± 1.08 g (mean ± SD).

The mice were housed in the Biomedical Laboratory facility in the pathogen-free, special care unit at the University of Southern Denmark and were acclimated for 1 week prior to surgery. The environment was controlled for temperature (21 ± 2°C), humidity (40–60%), and lights on between 6 a.m. and 6 p.m. The mice were housed individually in ventilated cages (IVC) with sawdust flooring and bedding material. They had access to sterile water and

to a control group with 5×10^5 MSCs and no VEGF for 8 weeks (Fig. 1).

Surgical procedure

All surgical procedures were performed at the Biomedical Laboratory at the University of Southern Denmark. The procedure followed protocol routinely used by the authors.[17]

Bone marrow was aspirated from five female donor sheep. This procedure was carried out under sterile conditions while the sheep were fully anesthetized with midazolam 1.0 mg/kg, and ketamine:midazolam at a 8:1 ratio for maintenance. Local analgesia of 5 mL (20 mg/mL) lidocaine was given s.c. at the aspiration site. Two incisions around the posterior superior crista iliac crest were made bilaterally, and 3–4 mL of bone marrow was aspirated using a bone marrow aspiration needle.

The aspirated bone marrow was cultivated in two passages. The MSCs were mixed with 40 mg (±1 mg) of HA, combined with 100 µL 10% FBS MEM medium in a syringe, and cultured overnight at 37°C, 5% CO_2. After culturing, HA and MSCs were implanted into the mice along with the rVEGF 165 pellets.

The mice were anesthetized with intraperitoneal ketamine (100 mg/kg BW) and xylazine (5 mg/kg BW) and supplemented with vaporized isoflurane (2%) in oxygen when necessary.

All components of the implants were injected by holding the syringes in desired locations approximately 1.5 cm from the incision site (Fig. 2). The operations were carried out between 12:00 p.m. and 6:00 p.m. in the pathogen-free, special care unit.

The mice were given analgesia in the form of buprenorphine (temgesic, 0.3 mg/mL) approximately 0.12 mg/kg BW s.c. every 8–10 h for the first 2 days after surgery.

Histomorphometry

Eight weeks postop, the mice were euthanized according to animal experiment protocol. The substitute and bone implants were harvested and placed in 4% formaldehyde and changed to PBS after 24 h. Paraffin was then embedded for classical histomorphometry of bone formation parameters. Each implant was split into four sections with 4 µm in thickness and a separation of 100 µm. The first three samples were stained with hematoxylin and eosin (HE) and the last section for human vimentin (hVim). The evaluation of bone formation was based on findings after HE staining and hVim staining.

The effects of the six VEGF groups were compared to the control. Volume fractions of each tissue in the predefined regions of interest (ROIs) were measured by Cavalieri's principle using stereological software (newCAST, Visiopharm, Denmark). The ROI was the TV of the entire section of each implant and gave approximately 500–800 points in each region for representable results.[18,19] The tissue within the ROIs of the HE stained sections were classified as bone (B), fibrous tissue (Fb), granula (Gr), miscellaneous (Mi), muscle (M), or marrow (Ma).

Serum biomarkers

Approximately 1 mL of blood was collected from each mouse at time of euthanasia (8 weeks). The blood was centrifuged for 10 min with 4000 relative centrifugal force (RCF) at 4°C to produce 500 µL serum. The serum was analyzed for bone markers to detect systemic activity of osteoblasts and osteoclasts. Osteocalcin was measured with ELISA Kit (Immutopics, Cat. #60–1305), pro-collagen type-1 (PINP) with EIA Kit (Immunodiagnosticsystems, Cat. #AC-33 F), carboxy-terminal collagen crosslink (CTX-I) with EIA Kit (Immunodiagnosticsystems, Cat. #AC-06F1), osteoprotegerin (OPG) with ELISA Kit (Biomedica, Cat. #BI-20403), soluble receptor activator and nuclear factor – κB ligand (sRANKL) with ELISA Kit (Biomedica, Cat. #BI-20462), and sclerostin with ELISA (Alpco, Cat. #41-SCLMS-E01) by following the instructions of each kit.

Statistical analysis

Unweighted one-way analysis of variance (ANOVA) was used to calculate overall differences between groups. *Post hoc* multiple comparisons were performed using the Holm–Bonferroni test as appropriate for normal distributions and Kruskal–Wallis test for non-normal distributions. p-Values $<5\%$ were considered significant. The statistical analyses were performed in GraphPad Prism v. 7 (GraphPad Software, Inc., La Jolla, CA).

In the sample size, calculation error of the first kind was set to 1.96/95% and error of the second kind to 0.84 due the selected power of 80%. Minimal relevant difference and standard deviation were both set to 70%.[20] The result was $n = 7.84$ implants; eight implants had to be included for each time point. We included four mice with a total of 12 implants in each group to account for the risk of illness or dropouts.

RESULTS

Animals

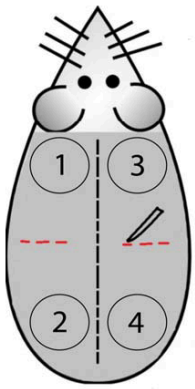
No mice were lost in this study. During the experiment period, they were observed daily by animal technicians or the researchers, and there were no violations of the animal license. All 28 mice were included although one mouse in the group with VEGF days 1–14 showed no bone formation in any of its implants and was excluded from further analysis.

CFU

CFU from five donor sheep was 41.3 colonies per 1×10^5 cells (SD ± 9.4). To ensure every group had homogeneous quality of cells, whether they were in the VEGF groups or in the control group, all MSCs were pooled before implantation.

Histology

Staining for hVim confirmed that the formation of new bone was from donor sheep MSCs and not the capability of the mice to generate bone subcutaneous (Fig. 3).



Groups	Number of implants	MSCs	VEGF
Control	12	5 x 10^5	None
VEGF release Day 1-7	12	5 x 10^5	8ng/day
VEGF release Day 1-14	12	5 x 10^5	8ng/day
VEGF release Day 1-21	12	5 x 10^5	8ng/day
VEGF release Day 1-42	12	5 x 10^5	8ng/day
VEGF release Day 7-14	12	5 x 10^5	8ng/day
VEGF release Day 21-42	12	5 x 10^5	8ng/day

FIGURE 1. Specified groups for the time points of VEGF stimulation and an illustration of the operation design with incision sites and numbered pouches.

Altromin total pathogen-free diet and were observed every day by either the animal technicians or the researcher for changes in behavior or signs of discomfort.

Sheep. Five skeletally mature female sheep (ewes), Texel/Gotland, were purchased 1 month prior to intervention so that they could acclimate to the environment. All sheep were of the same breed and origin, aged 5–8 years, and weighed on average 67 ± 14 kg (mean ± SD).

Animal approval. This study was approved by the Danish Animal Experiments and Inspectorate (no. 2012-15-2934-00704). The experiment complies with the national and institutional guidelines; the article complies with ARRIVE guidelines.

Study design

Subcutaneous implants—consisting of HA granules, MSCs from ovine bone marrow and a VEGF pellet—were prepared *in vitro* under general sterile conditions and collected in a 1 mL syringe. Two 0.5 cm incisions were made on each dorsal side of the mice, and the material was placed in 4 pouches subcutaneous (s.c.) at locations 1–3 (Fig. 1). After implantation, the mice were sutured, and the stiches covered with vetbond tissue adhesive glue (3M, St. Paul, USA).

Pilot study 1. The first pilot study was designed to indicate preferable MSCs concentration, mouse strain, and observation time to yield maximum formation on new bone formation.

In total, eight SCID mice were allocated randomly into two groups: four from Taconic NOD/MrkT (Cologne, Germany) and four from The Jackson Laboratory NOD.CB17-Prkdc^{scid}/J (Charles River Laboratories, France).

Pilot study 2. After looking at the results of pilot study 1, 5 × 10^5 MSCs were chosen. MSCs were seeded on the HA, inserted s.c. into four mice from The Jackson Laboratory NOD.CB17-Prkdc^{scid}/J (Charles River Laboratories), and observed for 8 weeks. The focus of this study was to find a release rate of VEGF which could influence the bone formation process without causing any noticeable side effects or lethal toxicity.

Primary study. Based on the pilot studies, a total of 28 SCID mice from The Jackson Laboratory NOD.CB17-Prkdc^{scid}/J (Charles River Laboratories) were used. The mice were randomly allocated to receive 8 mg/day VEGF by releasing the pellets at different time spans. The spans, days 1–7, days 1–14, days 1–21, days 1–42, days 7–14, and days 21–42, were combined with 5 × 10^5 MSCs and compared

FIGURE 2. Illustration of the timeline and procedures during the study.

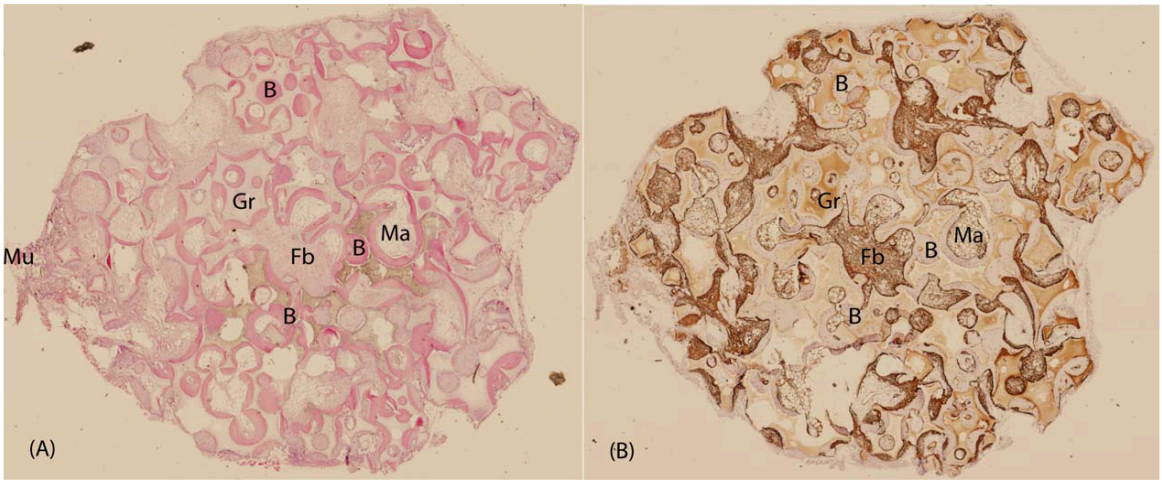


FIGURE 3. Histological images with (A) HE staining in group days 1–14. (B) hVim staining from the same implant. The distance between section (A) and (B) is 100 μm. B, bone; Fb, fibrous tissue; Ma, marrow; Gr, granula; Mu, muscle. The hVim positive areas are brown indicating that bone originated from the cultured cells. These overview pictures were taken with newCAST, Visiopharm, Denmark, lens ×4.

Histomorphometry

B/TV was significantly higher in the group with VEGF release on days 1–14 and days 1–21 when compared to the control group ($p < 0.01$). Days 1–7 and days 1–42 showed significantly more newly formed bone than days 21–42 ($p < 0.05$). No VEGF groups were worse than the control group.

Serum

VEGF groups were not different when compared to the control group (Fig. 4). The bone markers revealed a significantly higher P1NP and CTX on days 7–14 compared to days 1–42 ($p < 0.05$). OPG was higher on days 7–14 than on days 1–7 ($p < 0.05$), days 1–14 ($p < 0.05$), and days 1–21 ($p < 0.01$). Sclerostin values were significantly higher on days 7–14 compared to the other groups receiving VEGF ($p < 0.01$). RANKL was below the detection limit for the kit (62.5 ng/L) in all groups. Osteocalcin had no significant difference between any of the groups (Fig. 4).

DISCUSSION

This study investigated the effects of VEGF stimulation at different time points combined with MSCs seeded on HA granules in a subcutaneous (s.c.) mouse model for ectopic bone formation. The optimal time points for VEGF stimulation were days 1–14 and days 1–21 after surgery. hVim staining confirmed that generated bone originated from the sheep MSCs. The serum markers showed no difference which indicates this stimulation only had a local effect and no systemically detectable effect.

Previous studies in small animal models have proven the effect of VEGF[8–10] and the combination of stem cells and VEGF on bone formation.[6,11,12]

It has been demonstrated that the time span of administration of the VEGF protein is a substantial factor when attempting bone formation and should therefore be a consideration for the most efficient use of this protein in a variety of tissue engineering.

Because of the low *in vivo* half-life-time of the VEGF protein, different studies have used a variety of methods to prolong the release of VEGF. Due to the many different administration methods and release rates, it is difficult to compare the results from these studies and to replicate their results.

Several administration methods of VEGF have been used including postoperative injections,[10,11] dissolved hydrogel,[21,22] VEGF loaded alginate microparticles,[23] biodegradable porous systems,[8] and biomimetic bone matrix.[24] The variety of these methods suggests the need for an optimal VEGF delivery system which could be crucial when investigating the absolute effect of VEGF.

The VEGF release method used in this study was pellets with well-known release profiles (IRA). The release rate had been tested in a larger mouse study[25] to prove release rate over time. The conclusion was that daily secretion of the product might vary, but total product release during the desired time period was reliable.

To ensure that the newly formed bone originated from sheep MSCs and not the regenerative capability of the mice, the implants were stained for hVim. This staining identified the MSCs from the sheep due to cross-reactivity of the antibody against hVim with ovine but not murine vimentin. By comparing HE and hVim stained samples, we could confirm that the bone originated from the sheep MSC.[26]

The period of early bone formation is often defined as prior to 12 weeks. This study had an observation period of 8 weeks and was chosen according to pilot study 1. In mice euthanized after 4 weeks, the primary outcome in histological evaluation was clumps of cartilage cells.[12] A femur defect model in a rabbit showed no difference between 8 and 12 weeks.[8] Both studies used VEGF as a stimulator and supported the current findings of 8 weeks as a sufficient observation period for studying early bone formation.

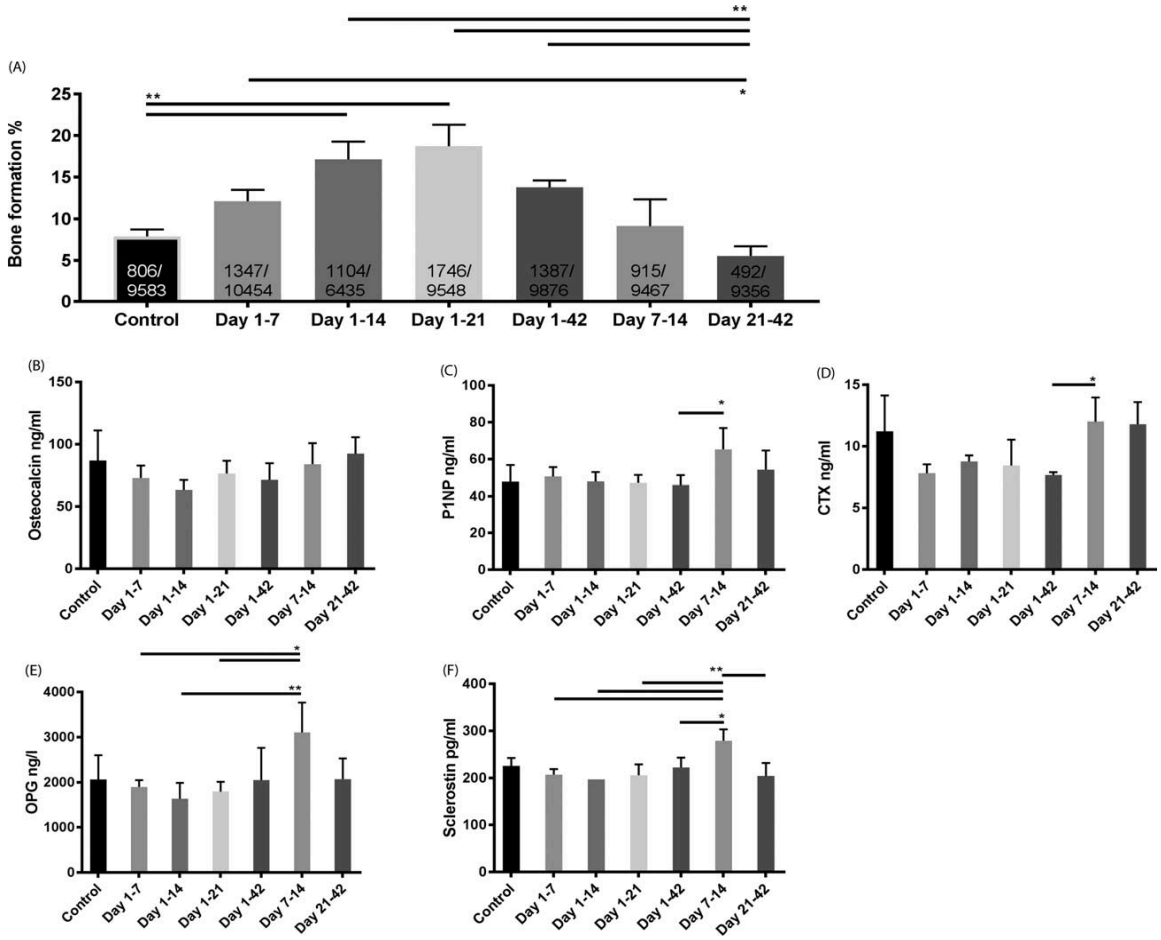


FIGURE 4. (A) Illustration of the bone formation (B/TV) in %. *x*/*y*; *x* amount of points for the counted new bone (B)/*y* the total amount of counted points from the entire group (TV). (B) Osteocalcin, (C) pro-collagen type-1, (D) carboxy-terminal collagen crosslink, (E) osteoprotegerin, and (F) sclerostin. $*p < 0.05$; $**p < 0.01$.

The natural secretion and systemic rise in VEGF upon fractures have been measured in both laboratory animals and humans. A natural peak in the systemic VEGF concentration in rats was measured on days 14 and 21 after fracture impact.[27] The same peak in human patients was on days 7 and 14.[28,29] This supports our findings for best bone formation and indicates that stimulation of VEGF should be increased during the natural systemic peaks postoperatively.

The strengths of this study were the standardized and reproducible methods (and thus the reliable results); and the cell culture procedures followed strict protocols. The VEGF pellets for the VEGF secretion have been used for >30 years and the release has been quantified and controlled (IRA). Every group consisted of 12 implants and would therefore be statistically significant according to the power calculation even if one mouse were excluded due to death or operation complications; moreover, we have many time points in the design to help narrow the duration for best use of VEGF.

Limitations in this study include the fact that some of the pellets were not fully dissolved at the euthanization point of 8 weeks. In these cases, the pellet area was excluded in the histomorphometry and should not affect results. The serum biomarkers in this study focused on the effect on bone formation and markers for osteoblast and osteoclast activity were prioritized. Unfortunately, we did not have enough serum to measure VEGF for a systemic rise; however, bone markers indicated that the VEGF from the pellets had no systemically detectable effect.

Future and ongoing studies in our laboratory will focus on a larger animal model, that is, a well validated implant gap model in sheep. These studies will quantify the efficiency of MSC and VEGF on new bone formation and implant formation in normal and osteoporotic sheep.

This technique of combining MSCs and VEGF with optimal release has the potential to accommodate the rising demand on the allograft bone for critical defects and could thereby reduce the hospitalization period for patients with greater bone loss.

Most bone formation was seen with VEGF stimulation on days 1–14 and days 1–21 during the ectopic bone formation in SCID mice with an observation time of 8 weeks.

Systemic serum biomarkers showed no change in bone markers due to the VEGF stimulation and did not cause concerns about systemic effects.

When put in perspective, a standardized surgical procedure for granules is suggested to ensure consistency of the substitute. Based on this study, it is suggested that future studies regarding bone formation with VEGF stimulation should consider the time point for optimal effect.

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Paper 2

The combination of vascular endothelial growth factor and mesenchymal stem cells for bone formation in a sheep model

Running head: Combination of MSC and VEGF for bone formation in sheep

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This article complies with ARRIVE guidelines.

Abstract

Introduction:
Mesenchymal stem cells (MSC) and vascular endothelial growth factor (VEGF) are two well-known factors in the field of bone regeneration. This osteogenic and angiogenic combination should, in theory, be able to establish a enhanced cell environment and attract bone-forming cells. This combination was used to evaluate bone growth in and around titanium implants compared to the golden standard of allograft.

Methods:
Eight Texel/Gotland mixed breed sheep were inserted with four titanium implants with a 2mm gap in each distal femur that was added autologous 3^x10^6 MSCs seeded on hydroxyapatite (HA) granules. Then, 0.015mg, 0.15mg or 0.525mg of VEGF was added to the implant neck. After 12 weeks, the implant blocks were harvested, divided in methylmalonic acid (MMA), embedded and sectioned or tested for mechanical strength (MTS), respectively. Blood samples were collected for the determination of bone-related biomarkers in serum.

Results:
The groups with the MCS and VEGF combination exhibited the same amount of bone formation within the gap as allograft ($p<0.05$). Moreover, no difference in MTS was observed between the groups ($p<0.05$). Serum biomarkers showed no significant difference to baseline.

Conclusion:
The bone-forming capabilities of the combination between MSCs and VEGF showed the same osteogenic results based on histology and mechanical strength in the trabecular structure of sheep, with no systemic increase in osteogenic angiogenic markers and generally no visible side effects over the evaluation period.

Keywords: Mesenchymal stem cells, vascular endothelial growth factor, bone formation, angiogenesis

1 Introduction

In the case of trauma and reconstructive orthopaedic surgery, the need for a consistent method of regenerating bone is a priority. The currently used allograft in the clinic has no osteogenic properties, carries a risk of disease transmission and it is a limited resource (1,2). Therefore, finding a biomaterial that has unlimited bone formation potential with no side effects would have a great impact on clinical outcomes, surgical complications and the economic perspective of shorter hospitalisation periods due to faster recovery, a lower risk of reoperations and, most importantly, improved quality of life for patients (3).

To create the optimal environment for tissue engineering, the need for neutral surroundings made by vascularisation is essential. Blood supply is one of the most common limitations in the bone regeneration cycle (4). Notably, the direct effect of vascular endothelial growth factor (VEGF) is the stimulation of blood vessels. It induces angiogenesis with the increased proliferation and migration of endothelial cells by enhancing vessel permeability and tube formation (5). The indirect effect of the VEGF is the initiation of mesenchymal stem cells (MSCs) into following the osteogenic lineage (6,7), which induces more osteoblasts at the healing site. While MSCs can be extracted from various tissues, MSCs derived from bone marrow (BMSC) have increased potency for bone regeneration with great potential in both animals and humans (8–10).

As a substitute for MSCs, hydroxyapatite (HA) has previously been highlighted as a suitable carrier (11). The rigidity and hardness of bone are due to the presence of mineral salt in the osteoid matrix, which is a crystalline complex of calcium and phosphate (i.e. HA). HA is ideal when compared to the human trabecular bone, which results in an osteoconductive scaffold. Even though it has been used over several years, it is still believed to be a promising factor within the field of tissue engineering (12).

The theory of optimising bone growth using a combination of MSCs and VEGF has previously shown bone formation *in vivo* (13,14). The problem when using these materials has been the question of administration, release and dosages (Study 3). A variety of methods for the local administration of VEGF have been investigated: from bioglass release (15) using cement (16) or prolonging the release with hydrogel (17). To this day, the optimal method for the release of growth factors is still being investigated.

The aim of this 12-week study was to determine the optimal dose of VEGF combined with MSCs in a bilateral distal femur implant gap model (*in vivo)*, *in vitro* and in normal sheep for new bone formation. MSCs were seeded on HA granules and implanted in the gap created by the titanium implant in the critical size defect (CSD). Prior to the initial study, one pilot study was performed to provide an indication of the most suitable number of MSCs for use in this model compared with VEGF.

The hypothesis of this study was that the effect of autologous MSCs on bone formation has the potential to be stimulated by an additional VEGF coating when compared to the golden standard of allograft.

2 Materials and methods

2.1 Animals

Sheep:
A total of 17 skeletally mature female sheep (ewes) of Texel/Gotland mixed breed were used for the pilot and initial study, and one was used for the *in vitro* pilot study for the aspiration of BMSC. All sheep were of the same origin and the study was performed during the summer with observed temperature and the possibility to be inside a stable or outside in a field. The sheep received hay and water *ad libitum* as well as regular dietary chips for sheep in calculated dosages. The sheep were aged between 5–7 years and weighed, on average, 74±22kg (mean±standard deviation (SD)). The acclimatisation period began one-month prior to bone marrow aspiration (according to animal guidelines by the Biomedical Laboratory, University of Southern Denmark) and animals were housed at the Biomedical Laboratory 5 days after surgery procedures.

Animal approval:
This experiment complies with national, international and institutional guidelines. The study was approved by the Danish Animal Experiments Inspectorate (no. 2012-15-2934-00704). Furthermore, the article complies with the ARRIVE guidelines.

2.2 Study design

Ti-6Al-4V implants made of 90% titanium, 6% aluminium and 4% vanadium at a size of 10mm x 12mm, with a 2mm gap, was inserted in a critical size defect in the distal femur (Fig. 1, T2 + T3). The implants were placed on both the lateral and medial side for a total of four implants in each sheep. Graft materials filled the 2mm gap of the implant and were isolated by a top washer to fixate the screw and material. The graft materials consisted of either the combination of MSCs and VEGF or the control of allograft.

MSCs were seeded on the HA and VEGF was added on the sand-dusted neck surface of the titanium implant at the aforementioned doses. Random allocation was only applied within each sheep; thus, every sheep contained one implant from each group (including control), though in different locations. The total observation for all *in vivo* groups in the pilot and primary studies was 12 weeks.

Pilot study

In vitro

This was performed to evaluate the influence of the coating materials and VEGF on the osteogenic capabilities of the MSCs. It was also performed to investigate the difference in using MSCs from normal or osteoporotic animal models. The study included three groups consisting of a group with 0.5×10^4 MSCs from normal or osteoporotic skeletally mature ewes, a group with titanium implants containing a PDLLA-VEGF coating with the release of 100ng/day, or combination treatment (Table 1).

This study followed the same differentiation protocol of the MSC as the *in vivo* portion. The cells were thawed and verified as confluent within 12 days and then added to VEGF implants. The evaluation was performed by alizarin red staining after 18 days of seeding. The staining indicated no difference when using either osteoporotic or normal MSCs (A1–B1). Positive reactions were observed in both the MSC alone and in the combination treatment, though no obvious differences were observed between the groups. VEGF alone had a very weak reaction to the staining.

Table 1

	Normal MSC	VEGF on titanium neck implant		Osteoporotic MSC	VEGF on titanium neck implant
Group A1 –	0.5 x 10^4	100ng release/day	**Group B1 –**	0.5 x 10^4	100ng release/day
Group A2 –	0.5 x 10^4	-	**Group B2 –**	0.5 x 10^4	-
Group A3 –	-	100ng release/day	**Group B3 –**	-	100ng release/day

In vivo

This pilot study was performed to indicate preferable MSC concentrations combined with VEGF for optimal new bone formation in 12 weeks. In total, eight skeletally mature ewes were used with the same surgical approach. The combination was 1^x10^6 cells, 3^x10^6 cells and 5^x10^6 cells on 150mg of HA combined with 0.015mg of VEGF, respectively. The control in this pilot study was 3^x10^6 MSCs without any VEGF. Furthermore, one implant was empty (without MSC or VEGF) and one had only 0.015mg of VEGF without HA or MSCs (Table 2). The pilot study indicated the same bone formation in the 3^x10^6 and 5^x10^6 MSC groups in combination with VEGF. Moreover, the empty implant showed no bone formation and characterised the defect as a CSD. VEGF alone exhibited sufficient bone formation in zone 2, though no ingrowth in zone 1.

Table 2:

	HA	MSC	VEGF
Group 1 – 8 implants	240mg	0.5 x 10^6	10ng release/day
Group 2 – 8 implants	240mg	1 x 10^6	10ng release/day
Group 3 – 8 implants	240mg	3 x 10^6	10ng release/day
Group 4 – 8 implants	240mg	5 x 10^6	10ng release/day

Primary study

A total of eight Texel/Gotland ewes were used. The groups were randomly allocated within each sheep to receive 0.015mg VEGF, 0.15mg VEGF and 0.525mg VEGF combined with BSA and

$3^{x}10^{6}$ MSCs seeded on HA for a total release of 10ng, 100ng and 500ng/release per day. (Table 3). Allograft served as the control group and was consistent in each sheep as its own control. The total observation period was 12 weeks.

Table 3:

	HA	**MSC**	**VEGF**
Group 1 – 8 implants	500mg	3 x 10^6	10ng release/day
Group 2 – 8 implants	500mg	3 x 10^6	100ng release/day
Group 3 – 8 implants	500mg	3 x 10^6	500ng release/day
Group 4 – 8 implants	500mg	Allograft	-

2.3 Vascular endothelial growth factor

Out of several VEGF family members, rhVEGF165 was chosen for the study. This VEGF was added to the neck of the implant and was not in correlation with the seeded HA. VEGF was combined with BSA at a ratio of 1:50 due to BSA having an positive effect on the stabilisation of the protein (293-VE, R&D systems), and it was delivered in a firm structure of 500ug. It was released by a poly-DL-lactic acid (PDLLA) (R203, Sigma-Aldrich) coating with verified release within 3 weeks based on a Bradford curve and sterilised with gamma irradiation by Synergy Health Radeberg GmbH, STERIS Germany before surgery.

2.4 MSCs from bone marrow

The autologous bone marrow was aspirated from the crista iliaca in four different areas for a total amount of 20ml. This was then transferred into four different 50ml falcon tubes containing 4ml of alpha minimum essential medium alpha (alphaMEMA, Life Technologies Europe BV, Denmark #22571-202) and 1ml heparin. After the aspiration, the tube was stored at +4^{o}C (Fig. 1, T1).

The bone marrow was diluted with phosphate-buffered saline (PBS, Dulbeccos, Life Technologies Europe BV, Denmark #14190094), alpha MEM, fetal bovine serum, (FBS, Sigma-Aldrich, Denmark #F0804) and penicillin streptomycin glutamate (PSG, Life Technologies Europe BV, Denmark #10378016). The colony-forming unit (CFU) cultivation was stained with crystal violet blue and counted 14 days after aspiration.

The subculturing of the MSC began when the cells had 80–90% confluence. Cells were prepared with PBS, trypsin-ethylenediaminetetraacetic acid (EDTA, 0.05%, Life Technologies Europe BV, Denmark #25300-054) MEM and then manually counted. The cells were then distributed in tubes of $5^{x}10^{5}$ cells for the preparation to be seeded on the HA.
For storage of the cells, 10ml syringes were cut open at the tip and placed in upright positions in the intubation chamber during the night before surgery. The syringes were then filled with 500mg (± 1mg) hydroxyapatite and then added to a 100ul growth medium and cell suspension with $3^{x}10^{6}$ cells (11).

The cells were verified as plastic-adherent. No immunohistochemical analysis was performed. These are termed multipotent mesenchymal stem cells by the International Society for cellular therapy (ISCT) and will be referred to as MSC in this paper.

Figure 1:

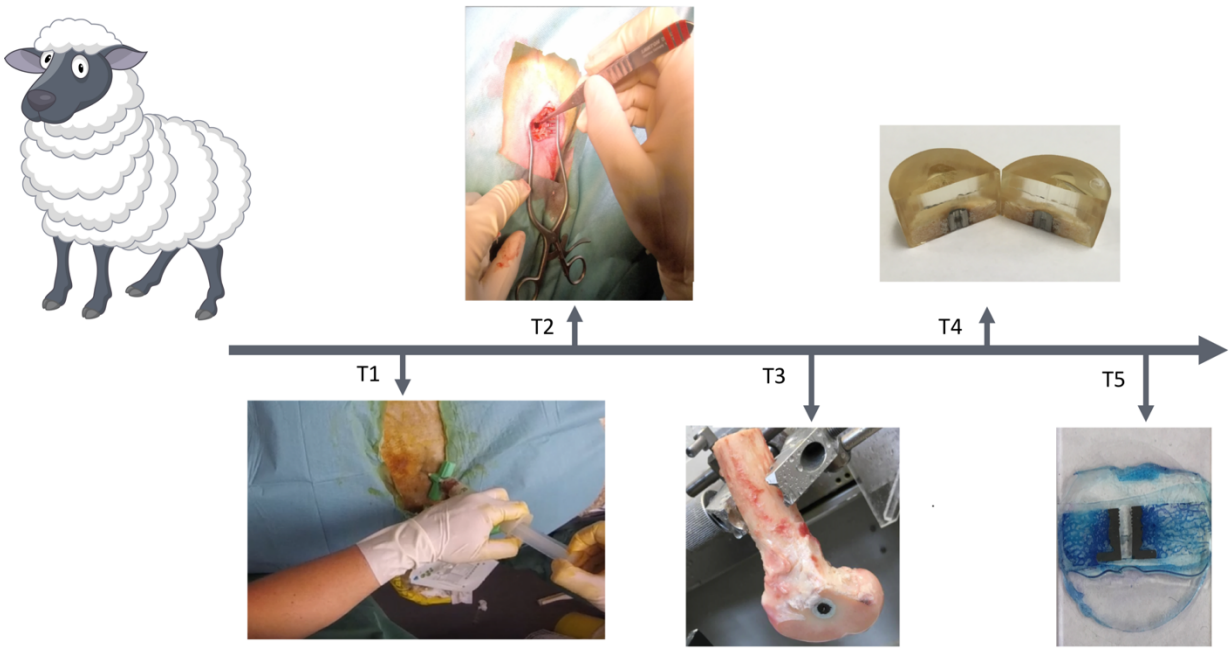

Figure 1: *Illustration of the order of intervention. T1 (week 0): Aspiration of bone marrow for the cultivation of MSC. T2 (week 4): Surgical femoral implant gap model. T3 (week 16): Preparation of samples for MTS and embedding. T4 (week 28): Samples embedded and ready for sectioning. T5 (week 29): Samples stained for calculation.*

2.5 Colony forming unit

After the cells were diluted and isolated, 0.5–1 mL of cell suspension was prepared for CFU staining. A total of 1×10^5 cells were then evaluated for CFU cultivation. The counting procedure followed previous guidelines using a BX47 microscope (Olympus, Tokyo, Japan) with a 4x lens (18).

2.6 Graft material

The substitute used as a carrier for the MSCs consisted of pure HA granules. The diameter of each pore was between 1.0–2.5mm (ENGIPORE, Fin-Ceramica, Faenza, Italy). This size would reach ~90% relative to the total volume and be transferred to the gap model in all sizes.

2.7 Surgical procedure

All surgical procedures were performed at the Biomedical Laboratory at the University of Southern Denmark.
Before handling, animals received 0.01ml/kg of Rompun (xylancin hydrochloride, 20mg/mL; Bayer Animal Health GmbH, Leverkusen, Germany). Bone marrow aspiration was performed under general anaesthesia with 3 mg/kg of propofol (10mg/mL; B. Braun), while surgical procedures were performed under general anaesthesia (2.0 % isoflurane) using aseptic techniques methodology including ethanol 70% and iodine vet (Kruuse) for disinfection.

Bone marrow aspiration was performed autologously from all sheep. The procedure was carried out under sterile conditions. Local analgesia of 5mL (20mg/mL) lidocaine was applied s.c. at all four aspiration sites. A bone marrow biopsy aspiration needle (13gax2-1/2in, Angiotech) penetrated the skin at the four sites located laterally from the spina iliaca posterior superior crest, and 3–4ml of bone marrow was aspirated bilaterally (Fig. 1, T3).

In the femoral gap model, the primary incision was placed at the lateral or medial condyle site, and the periosteal surface was exposed by an incision through the fascia with electrocauterisation splitting of the soft tissue. A low-speed drill created a 12mm-deep cylindrical hole with a circumference of 10mm. To remove residual bone particles, the gap was rinsed with saline before insertion of the implants forming a gap of 2 mm. Subsequently, the concentric gap was blindly allocated to one of the four groups, the gap was filled with substitute and the top washer of the

implant was fixated. Finally, the wound was sutured in three layers and wound plast was added (with tar, Kruuse). Postoperative analgesia and antibiotics included 0.3mg/mL buprenorphine (Temgesic) and Curamox (150 mg/mL amoxicillin), which was administered daily for 3 days. After 12 weeks, the sheep were euthanised with Euthanimal (200mg/ml, Alfasan) and distal femurs were harvested bilaterally and further processed based on an existing protocol (19).

2.8 Tissue handling

Twelve weeks after surgery, the sheep were euthanised. The implant blocks with surrounding gap bone were divided into two parts using Exakt diamond band saw (Exakt Apparatebau): one 6.5mm-thick sample was dehydrated in graded ethanol (70–99%) at room temperature with electronic stirring and subsequently embedded in methyl methacrylate (Technovit 9100). After sectioning, the glasses were stained with toluidine blue O staining for histological analysis. Another 3.5mm-thick sample was stored at -20° for mechanical push-out testing.

2.9 Histomorphometry

Polarised light was used to determine the placement of the collagen lamellae to distinguish between mature bone with regular alignment compared to randomly oriented collagen fibres and cell lacunae. Volume fractions of each tissue in the predefined regions of interest (ROI) were measured by Cavalieri's principle using stereological software (newCAST, Visiopharm, Denmark). The ROI was the 2mm gap (TV) of four sections from each implant and gave approximately 2000–3000 points pr. implant for representable results (20,21). Furthermore, the gap region was divided into two sections: close to the implant (zone 1) and close to the existing bone (zone 2). The tissue within the ROIs of the toluidine blue O-stained sections was classified as bone; blue coloured as osteocytes and fibrous tissue; purple with visible fibril fibres and low cell density as granula; black as the implant, miscellaneous, or marrow; and non-stained areas as adipose (Fig. 3). Blinding of the intervention group and control in the evaluation were difficult due to the histological characteristics of HA; however, interventions groups still could not be distinguished.

Figure 2:

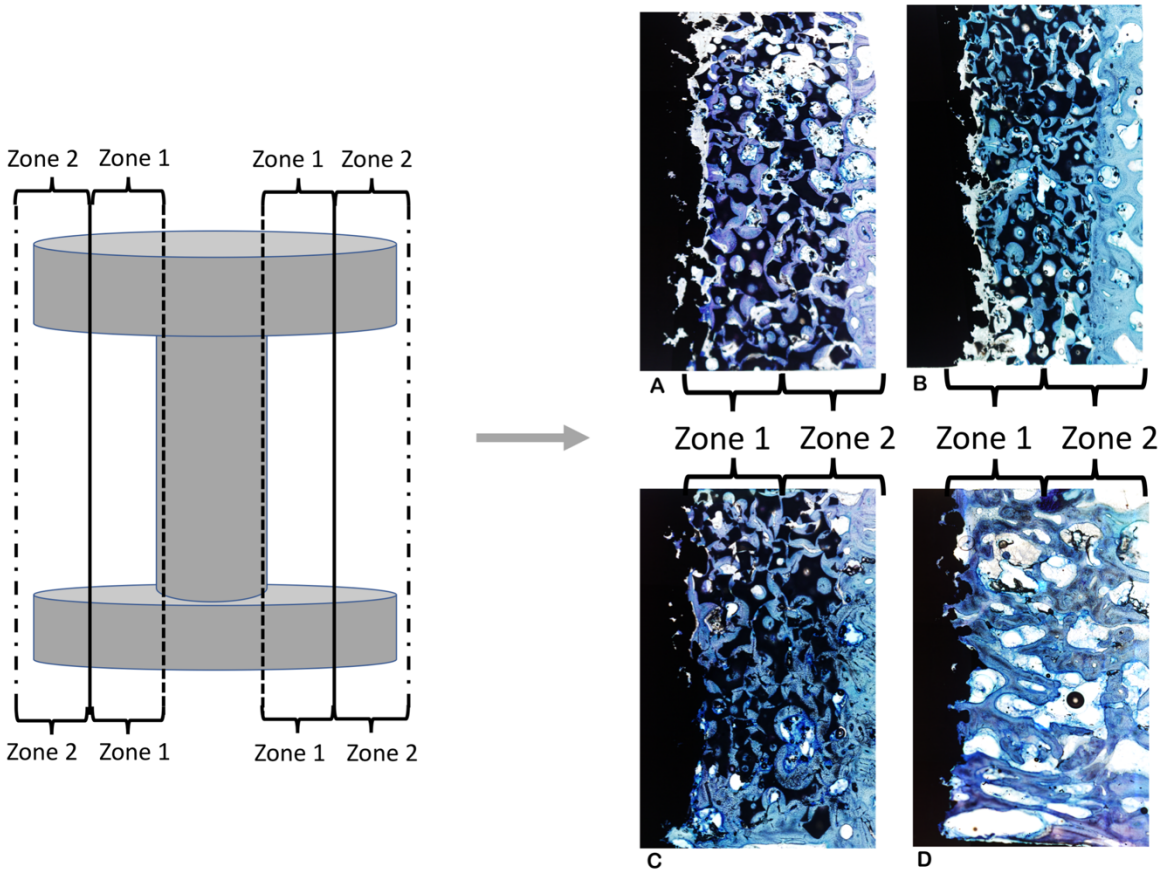

Figure 2: *Illustration of the implant divided into zone 1 (close to the implant) and zone 2 (close to the existing bone). Histological images from the gap in each group were stained with toluidine blue O after 12 weeks. A: MSC+VEGF 10ng/day. B: MSC+VEGF 100ng/day. C: MSC+VEGF 500ng/day. D: Allograft. These overview pictures were taken with newCAST (Visiopharm, Denmark; lens 4).*

2.10 Serum biomarkers

Approximately 20 ml of blood was collected from the jugular vein at different time points: at surgery day, 1 week, 2 weeks, 4 weeks, 8 weeks and at euthanisation (12 weeks). Since the containment of MSCs and VEGF were the same within each sheep, results are compared to the preoperative baseline for any systemic effect. The blood was centrifuged for 10 minutes with 4000 relative centrifugal force (RCF) at 4ºC to produce 8–10ml of serum. The serum was analysed for bone markers to detect the systemic activity of osteoblasts, osteoclasts and VEGF to qualify any systemic effect from the stimulation during the early bone regenerative phase. Osteoprotegerin (OPG) Sheep OPG ELISA kit. Cat no: MBS2506141, receptor activator and nuclear factor – jB

ligand (RANKL) Sheep Receptor activator of Nuclear Factor KB Ligand (RANKL) ELISA kit, pro-collagen type-1 (PINP) Sheep Pro-collagen Type I N-Terminal Propeptide (PINP) ELISA kit, Sclerostin, Sheep Sclerostin (SOST) ELISA kit, Cat no: MBS033198, VEGF Sheep Vascular Endothelial Growth Factor. Cat. No: MBS737944, fructosamine Sheep Fructosamine ELISA kit, Bioassay Technology Laboratory, Cat. No. E0122Sh and osteocalcin and carboxy-terminal collagen crosslink (CTX-I) by iSYS immuno-diagnostic-systems IDS.

2.11 Mechanical testing

Following storage at -20°, the samples were placed in room temperature for 2 hours prior to mechanical testing. Then, 3.5mm bone blocks containing implants were placed under a 6mm piston from the Mechanical Testing System (MTS, hydraulic material testing system; MTS Systems Co). Using force, the piston measures the force in newton/area compared to the displacement of the implant with a placement rate of 5mm/min. This provides measures of shear stiffness (MPa) and total energy under the curve for failure (kJ/cm^2) at the maximum power (MPa) provided for bone breakthrough (22).

2.12 Statistical analysis

One-way analysis of variance (ANOVA) was used to calculate overall differences between groups and compared to the control (allograft). Multiple comparisons were performed using the Holm-Bonferroni test (as appropriate) for normal distributions and the Kruskal-Wallis test for non-normal distributions. *P*-values less than 5% were considered significant. The graphs and statistics were measured and constructed in GraphPad Prism v. 7 (GraphPad Software, Inc.). The error of the first kind ($t2\alpha$) was set at 1.96 with a confidence level of 95%. The critical value for the error of the second kind (t_β) was 0.84 due to the selected power of 80%. The minimal relevant difference was selected at 70% and the SD at 50%. According to these assumptions, at least six samples should be included in each group. We included eight sheep (i.e. 8 implants) in each group to account for any illness or dropouts.

3 Results

3.1 Animals

The animals were observed daily by animal technicians. All sheep survived, and no sheep showed sign of any illness or stress during the experiment. The total of four implants did not contain any HA in the histological sectioning, and these were excluded due to a lack of MSCs. No group had less than six samples according to the power calculation.

3.2 Colony forming unit

The CFU from all donor sheep was 51.3 colonies per $1x10^5$ cells (SD ± 15.1). The bone marrow was aspirated and implanted autologously.

3.3 Histology

Staining with toluidine blue O showed general new bone formation within the entire gap of the implant. Furthermore, there was ingrowth to both the implant and existing bone for promising osteointegration (Fig. 2).

3.4 Histomorphometry

The evaluation of bone volume within the gap showed no significant difference between either of the intervention groups or between the allograft (control) group ($p<0.05$) (Fig. 3). In zone 1, the 100ng/VEGF group had significantly lower bone formation than the allograft group ($p<0.05$) and significantly higher bone formation in zone 2 ($p<0.001$). The surface calculation showed no difference to the control ($p<0.05$).

Figure 3:

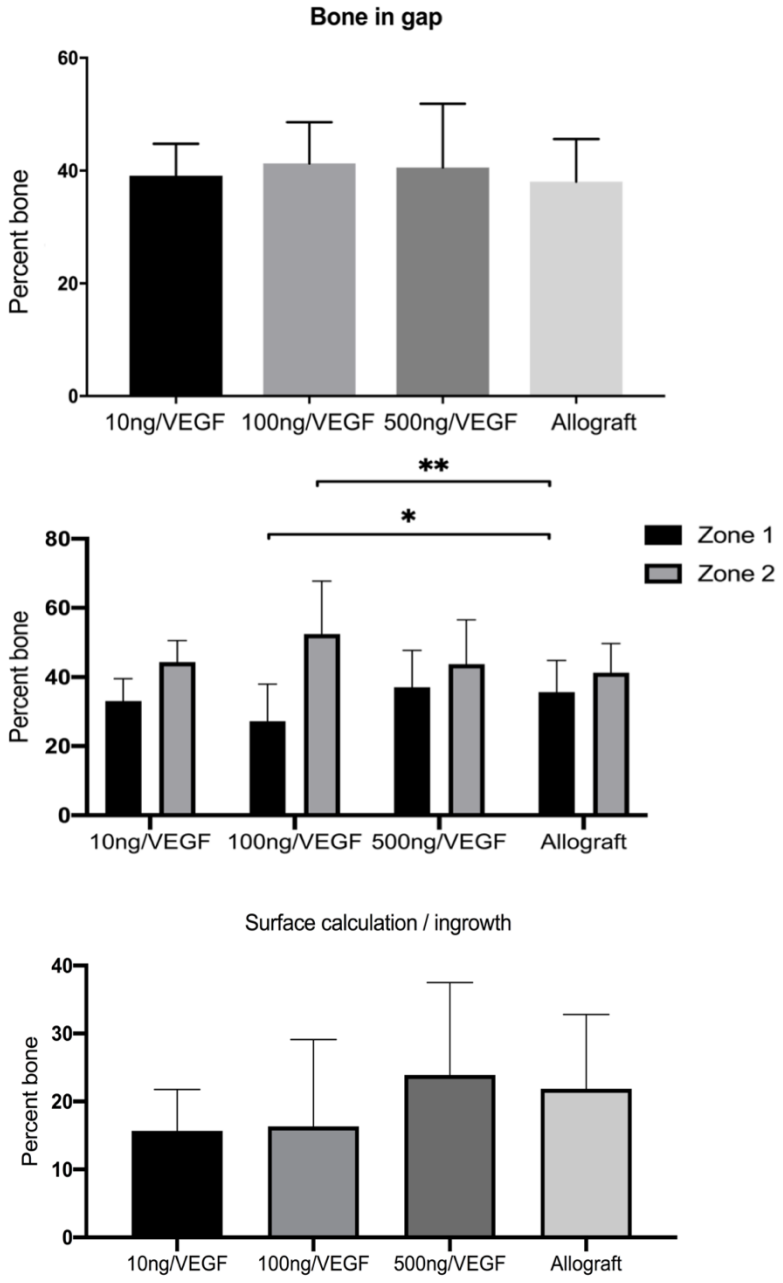

Figure 3*: Percentage calculation of bone within the gap based on histological images. No statistical differences were observed between the groups in overall bone within the gap or in the bone ingrowth. 100ng/VEGF had a lower percentage in zone 1 compared to allograft (*p<0.05) and a higher percentage in zone 2 (** p<0.001).*

3.5 Serum

The bone markers revealed no significant differences between the baseline and the measured time

points. However, a tendency of decreasing VEGF and RANKL was observed after week 1 with increasing osteocalcin and CTX. Moreover, VEGF exhibited a significant decrease from week 1 to week 8. Sclerostin could not be measured by the provided kit and was thus excluded from the analysis. OPG values were all below the minimum for the kit (i.e. <0.78pg/ml).

Figure 4:

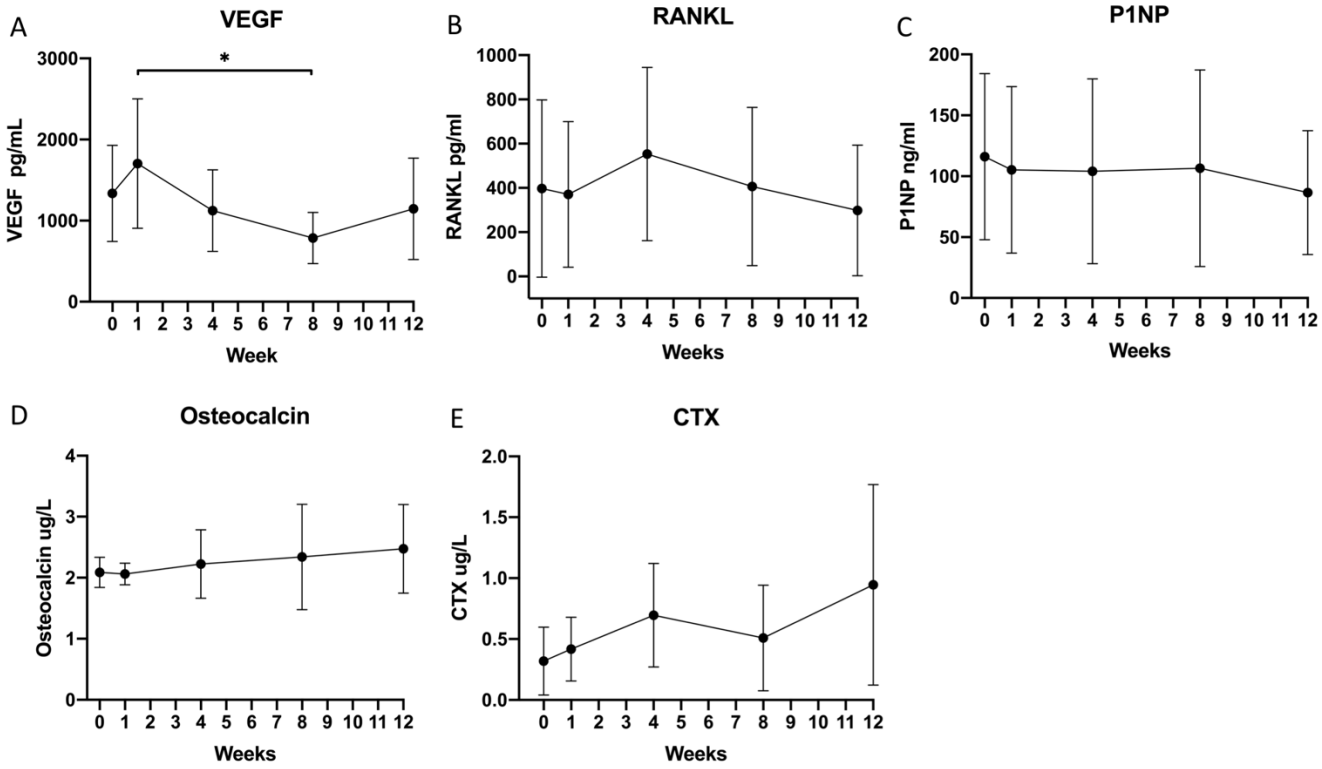

Figure 4: *Blood serum samples at different time points. A: Vascular endothelial growth factor (VEGF) B: receptor activator and nuclear factor – jB ligand (RANKL). C: pro-collagen type-1 (P1NP). D: Osteocalcin. E: carboxy-terminal collagen crosslink (CTX). *p<0.05.*

3.6 Mechanical testing

Mechanical testing was applied in the same direction as the neck and the screw to evaluate the ingrowth and strength of the implant into the newly formed bone. This evaluation revealed no significant differences in shear stiffness, shear strength, or failure energy ($p<0.05$) among the groups. This suggests that the quality and ingrowth of the bone have equal properties to those of allograft.

Figure 5:

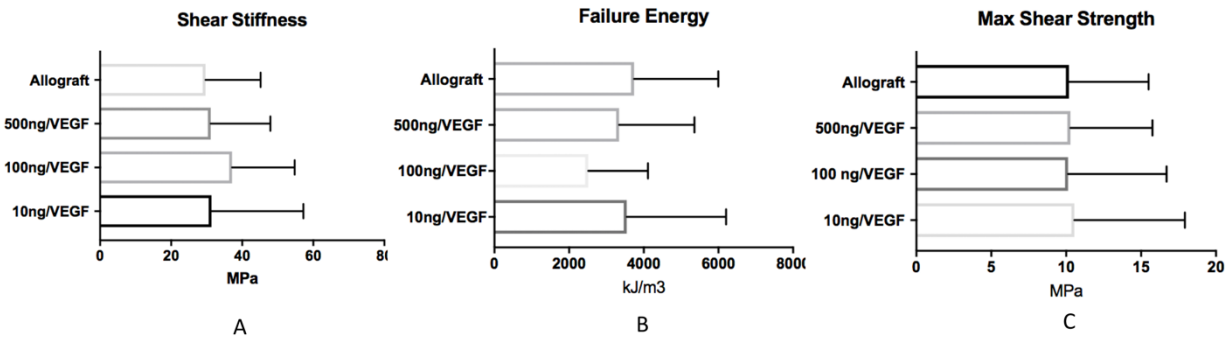

Figure 5: *Mechanical testing from all groups defined by three parameters: shear stiffness, failure energy and max shear strength. MPa (MegaPascal), kJ/m^3 (Kilojoule/cubic metre). No statistical differences were observed between each group in any of the parameters.*

4 Discussion

4 Discussion

This study investigated the effects of the combination of MSC seeded on HA and VEGF in a critical size defect model in trabecular bone structure for bone formation in the sheep model in comparison allograft after 12 weeks. No statistical differences were observed when combining 3^x10^6 MSCs with 0.015mg VEGF, 0.15mg VEGF or 0.525mg VEGF, or only using allograft, in histological sections or mechanical testing. Moreover, serum samples showed no systemic effect of the stimulation.

The use of MSCs and VEGF has existed for bone formation in the past, and the research community continues to seek the optimal strategy for optimal bone formation in critical size defects (13,23). While factors such as BMP-2, BMP-7, IL-1, IL-6, TGF, and FGF at different dosages and combinations have been attempted using different methodologies, they have exhibited a weak impact on clinical procedures. Furthermore, the use of various stem cell types (e.g. bone marrow stem cells (BMSC), adipose-derived stem cells (ADSC), muscle-derived stem cells (MDSC), embryonic stem cells (ESC), etc.), different types of scaffolds (e.g. 3D printing, titanium,

magnesium, etc.) and indirect stimulation with mRNA, calcitonin gen-related protein (CGRP) or signalling pathways (e.g. Wnt) have also been attempted. The possibilities related to the absolute effect on bone defects are endless (24,25). Many studies are being performed on rodents or rabbit with good effect, though these studies and strategies are never published for larger animal studies (13,14,26). This trend could be due to research groups not having the option to perform these studies or that the results, when used in the larger animals, were insufficient.

Firstly, we attempted the combination and the method of cultivating and verifying the plastic-adherence capabilities of MSCs in a mouse model in combination with VEGF (11). Then, we attempted to translate this design into a larger animal model that showed the same bone-forming quality and quantitative effect as allograft to further explore the opportunity for use in translational research. The difficult part of working with VEGF is the release, which is due to its short *in vivo* half-life. This was combined with the low reproducibility of results, which might affect the total dosage and release period in existing designs Study 3+ (27). This negates the need for a suitable carrier and reasonable dosage when used for protein to have an optimal effect on bone formation.

The dosages of VEGF used was inspired by a previous design in SCID mice, and the same dosage of 0.015mg VEGF combined with BSA (11) were used in this design. The other dosages were x10 and x25 of this initial dosage due to the translation into a defect in a larger animal model. Surprisingly, there was no statistical difference between the groups of different VEGF dosages. This could either be due to the different quality of stem cells or the dosage range of VEGF in this design being quite wide. In our pilot study, the control was $3^{x}10^{6}$ MSCs without any VEGF, but with only 240mg of HA. Here, the MSC and VEGF combination exhibited higher bone regeneration than MSCs alone.

The methodology in these designs is very dependent on being simple, feasible and cheap in order to increase the chance of implementation (28). Working with differentiated stem cells requires a greater focus on elective procedures rather than acute surgery. This gap design does not indicate any specific clinical issue but provides a proof of concept effect of the trabecular bone structure, which is well represented in the spine, for example. Furthermore, due to the strong nature of HA, this design can provide instant stability (29). The titanium implant has a porous plasma-sprayed structure that is similar to the general stems and cups used in arthroplasties. This makes the design feasible for increased bone ingrowth and less stress shielding.

The strengths of this study were related to its reproducible design. Our research group has made the femoral gap model several times and thus enhanced the quality of operation and evaluation each time (19,22). Moreover, cell aspiration and cultivation followed strict and reproducible protocols. The handling of the MSCs in all our studies was performed using the same experienced leading laboratory technician. The blood sampling did not focus on specific dosages for a systemic effect; instead, it explained the systemic reaction in the development of bone growth and release of all of MSC and VEGF to verify possible uses for further translational studies.

The limitations of this study included the low amount of test subjects and implants, which reduced the power of the results. Hence, this study meets the power calculation and further attempts to consider the 3 Rs in the model by using several implants per sheep. Furthermore, the amount of HA required to fill our gap was increased by two from our pilot study to the initial study. Unfortunately, some sections did not contain any HA with MSCs and were thus excluded from the results section. This result could be due to variation in granule size as opposed to the total weight of the substitute, which should be considered when using different granule sizes.

Regarding the future, we have ongoing studies that will use this model in osteoporotic-induced sheep to focus on the bone structure in the older human population. These studies will quantify the efficiency of MSCs and VEGF on new bone formation, even in the context of poor bone quality and a decreased angiogenic environment for bone growth. The potential for this model in terms of correct dosages and usage has shown its potential for clinical use as an alternative to allograft.

In conclusion, based on histology and mechanical testing results, combining MSCs and VEGF in the femoral gap model in the trabecular bone has the same capabilities as allograft for bone formation after 12 weeks. Moreover, systemic serum biomarkers showed no change in bone markers compared to baseline at any studied time point and level of VEGF stimulation. There were also no concerns regarding systemic effects.

The results of this study illustrate the need for standardised procedures regarding the optimal use of bone enhancing materials, with a focus on the possibility of translational medicine. Based on our results, we suggest that future studies consider the combined effect with VEGF to stimulate the angiogenic environment in bone growth.

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Author Disclosure Statement

All authors state that there is no competing financial interest exist.

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Paper 3

The use of vascular endothelial growth factor for in vivo bone formation: A systematic review

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Abstract

This systematic review recapped the last 10 years of *in vivo* bone regeneration using vascular endothelial growth factor.
A total of 1374 articles were identified. 24 articles were included. 11 articles used VEGF alone and had a group or a timepoint better than the control group. 18 articles with no significant difference to control and 1 article reported decreased bone growth.
Based on these results indications could be made towards the optimal release of VEGF is within the first three weeks, in defect models, with optimal effect before eight weeks. Future designs should incorporate this with standardised and reproducible models.

Introduction

Bone loss and defects is a very specialised area in the field of orthopaedics, and knowledge of the physiological parameters in bone formation is subject to an ongoing investigation. Research in bone formation has attracted increasing interest due to an increasing elderly population and fracture rate, where a significant percentage of the fractures have inadequate defect healing due to infections, surgical procedures and more fragile bone structure (1,2). One well-known factor in achieving sufficient bone healing is to secure sufficient vessel contribution to the defect, with some research on growth factors focusing on the use of the angiogenic protein vascular endothelial growth factor (VEGF).

VEGF is of particular interest in bone regeneration due to its primary ability to induce neovascularisation (10,15,22). The VEGF protein also carries the indirect ability to differentiate MSCs into the osteogenic lineage (3,7). Furthermore, it has a chemokine effect on surrounding endothelial cells (ECs) that can increase the number of vessels in a localised area (8). These effects contribute to achieving a more controlled use of both the stimulating effect of VEGF and regenerative effects of the MSCs. Notably, the inhibition of VEGF has led to non-union models (9), making the growth factor essential in the bone healing process.
The VEGF family consists of different members, including VEGFA, -B, -C and -D, and PIGF1 and 2 (8,10). The most commonly used isomer of VEGF in the field of bone research is the human VEGF165 (rVEGF165)—a member of VEGFA—due to its elevated potency and effect (24).

Bone formation can be divided into intramembranous and endochondral ossification. The primary stimulator of VEGF expression in the osteoblast-like cell line is hypoxia-induced factor 1α (HIF-1α), while the release of HIF-1α is related to the initiation of fracture repair in the endochondral ossification (12,13). The initial stage of endochondral bone formation is the cartilage base structure created by the osteochondral progenitor cells within regions of low blood perfusion. Then, the chondrocytes proliferate and enlarge. This initiates the attraction and incorporation of endothelial cells, osteoblastic precursor cells, haematopoietic cells and osteoclasts that results in the cartilage becoming degraded and replaced by trabecular bone and bone marrow (14,15).

Generally, abundance of blood vessels around the healing site will enable more nutrients and regenerative cells to be transported from the bloodstream to the area of bone formation, with more waste products from the healing site being moved away from the desired location (16). However, the VEGF protein appears to have a narrow therapeutic window (4), which implies that if the dosage of VEGF is too high, this can cause malformed and non-functional vessels as well as a potentially toxic effect (4,17).

Furthermore, VEGF has a half-life between 4–24 hours (18,19). This short period makes it difficult to use in the surgical setting if a long-term effect on the defect is desired without reaching the toxicity threshold. This issue has caused multiple delivery methods in experimental designs to prolong the release of VEGF.

Theoretically, the VEGF protein should have the capability to enhance the regeneration of bone defects, especially endochondral ossification. The growth factor is already applied in human clinical trials for other purposes (20) and would have the natural capability to transition to the field of bone research. However, to our knowledge, clinical trials using VEGF in this field have not yet been published.

This systematic review aspires to collect all existing *in vivo* results on the solitary use of vascular endothelial growth factor for bone growth compared to control, as evaluated by new bone volume/tissue volume (BV/TV). Additionally, we evaluate whether these results indicate any promising progress towards release methods and dosages that could be applied in a focused experimental design and translated into human clinical use. Our hypothesis is that solitary VEGF stimulation in the current *in vivo* literature will demonstrate a pattern and method for human application in bone formation.

The translational potential of this article

This systematic review aims to assess the existing literature to focus on methodologies and outcomes that can provide future knowledge regarding the solitary use of VEGF for bone regeneration in a clinical setting.

Keywords: Angiogenesis, Biomaterials, Osteogenesis, Growth factors, Tissue engineering, Vascular endothelial growth factor.

Materials and methods

Design

Inclusion criteria include *in vivo* studies in both animals or humans using VEGF compared to empty defect or empty control, where the outcome measure is bone growth within a region assessed either by micro-CT or histomorphometry. All human studies were included. If a study used VEGF in combination with other hormones or growth factors, only the VEGF-only groups were included. Articles were restricted to English articles with full text available that were published during the last ten years (Fig. 1). Exclusion criteria were all *ex vivo* studies, reviews, explanatory articles, conference abstracts, lectures and newspaper articles.

A ten-year duration was selected due to an existing systematic literature review produced in 2008 on the effect of VEGF on bone formation (21). The latest included article from this study was from 01-11/2007.

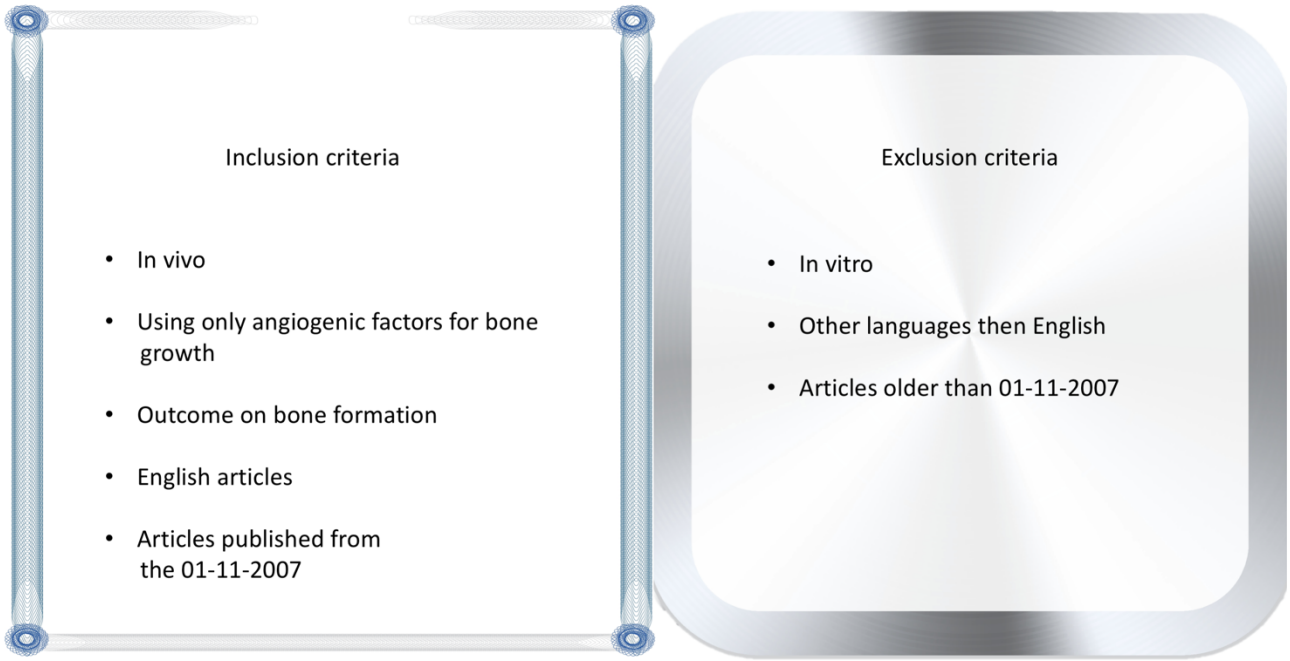
Studies were identified through PubMed using the search string *(vegf or "vascular endothelial growth factor") and (osteogen* or "bone formation" or "bone regeneration")*. Studies were then filtered for full text only, human or animal studies, and publication date from 1 January 2007 to 01 July 2019. Studies were then screened by title and abstract, and the full texts of eligible studies were analysed for final inclusion.

Study selection was divided into different phases. In phase one, articles were evaluated by their title and abstract. In phase two, articles were assessed based on their full text by primary inclusion and

exclusion criteria without any evaluation of results. In the final phase, relevant studies were subject to quality assessment using a scoring system.

The following data were extracted from the included studies: journal type (peer-review or not), animal model details, tissue type (e.g. cranial or ectopic bone formation), type of scaffold, type of control group, type of randomisation, type of VEGF and its release system, justification for VEGF dose and/or release rate, experiment duration, type and magnitude of outcome, type of blinding, sample size considerations, and ethics and disclosure statements. The corresponding author of the study was contacted if any of the aforementioned information was missing from the articles. Data extraction was performed by CHD and verified by KK. At any disagreements, the third or fourth authors were included and a discussion was initiated until agreement by all authors.

Figure 1



The general inclusion and exclusion criteria

Assessing the risk of bias among articles was inspired by Macleod et al., while the pooling of animal experimental data was provided by CAMARADES for experimental animals (22) (Table 1). This assessment consisted of 10 items, with each providing 1 point. Three items were removed and two items were added to highlight the hypothesis of this systematic review. This meant that questions regarding the control of temperature, the blinded induction of ischaemia, and the use of anaesthetic without significant intrinsic neuroprotective activity were removed. Instead, the justification of a control group and VEGF dosage were added.

The total assessment of the risk of bias was based on scientific quality according to peer-reviewed articles, control groups, random allocations to intervention groups, blinded evaluations and sample size calculation. Furthermore, the reproducibility of dosage and animal models, any conflict of interest, and animal regulations were also considered.

Statistics were calculated by one-way ANOVA for the comparison of different parameters, when applicable. A p-value of less than 0.05 was considered significant.

Results

A total of 1374 articles were identified using the PubMed search. During the title/abstract screening, 1304 articles were excluded, leaving 70 articles for the full-text analysis. The full-text analysis excluded 46 articles: 1 used a modified adenovirus, 5 were *ex vivo* studies, 21 were missing bone volume/tissue volume evaluation and 19 articles did not have a group with the solitary use of VEGF for bone formation (Fig. 2).

Figure 2

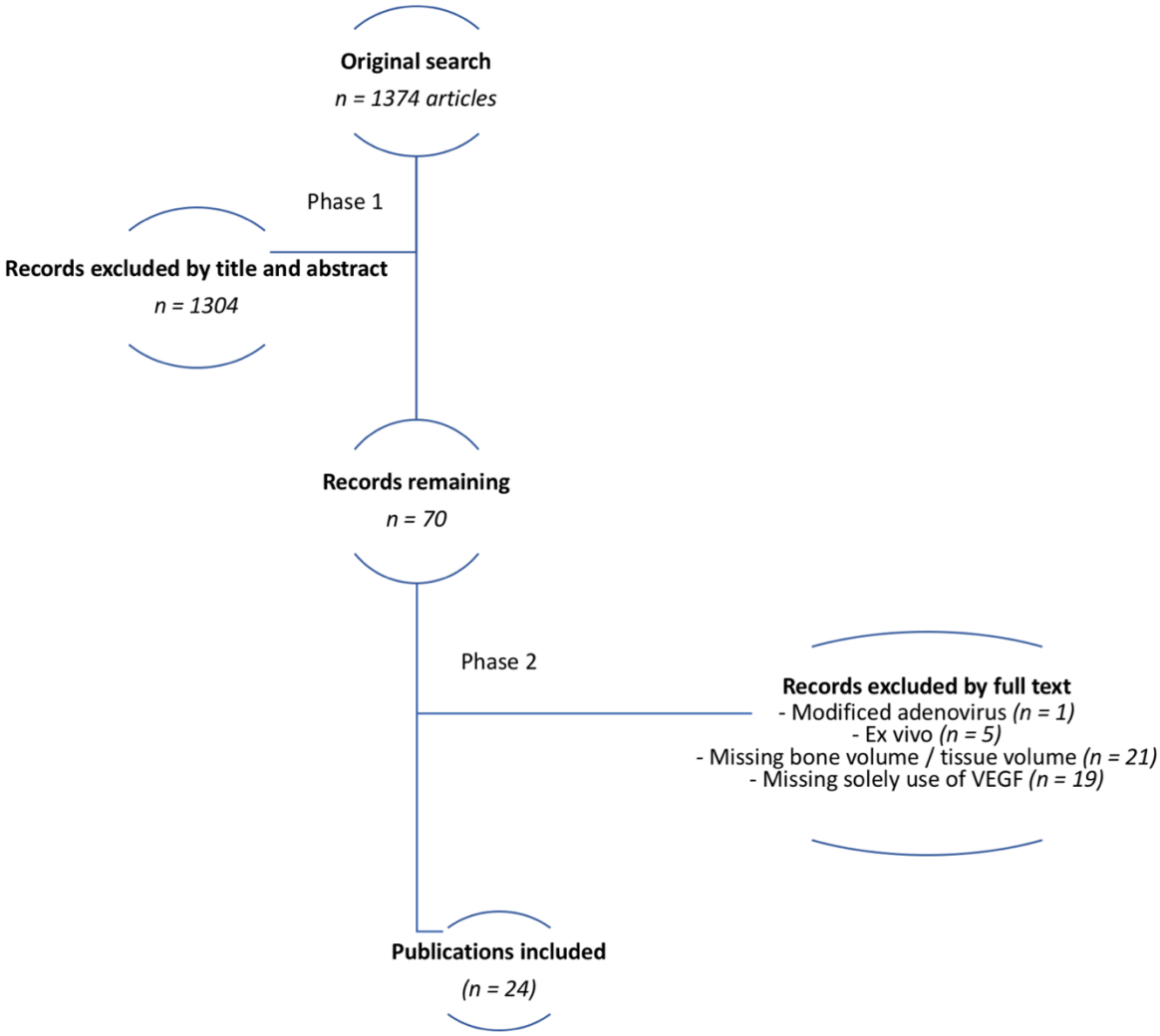


Illustration of the search strategy for the systematic review. A total of twenty-four articles were included.

The content of each article is illustrated in Table 2. No studies used VEGF in human models. The experimental models were as follows: rats (23–31), mice (32,33), rabbits (34–41), beagles (42–45) and pigs (46). The types of defect were as follows: cranial/calvaria/skull defects (23,26,29,30,33,42,46), intra-femoral implantation (32), mandible (24,31,34,39,41,43,45), femoral neck fracture model (42), femoral condyle defect (35,38), femoral shaft defect (28), ectopic (25,44), sinus floor elevation surgery (37), tibia head implant (27) and radial diaphysis (40).

VEGF was released by different compounds as follows: gelatine (28,29,32,34,44), plasmid DNA (32,41), poly-d,l-lactic acid (PDLLA) (24), fibrin glue (35,40,42), PLGA microspheres (30,31,38,43), PDLLA/CO3 (25), silk scaffold (36), hydrogel (37), alginate microparticles (26),

PLGA microparticles (44), DNA oligonucleotide (27), Bio-Oss (46), hydroxyapatite (45), collagen sponge (33) and injection (39).

The dosage of VEGF was mentioned as either a total dosage, a dosage per area, or an amount in fluid. Doses were as follows: 0.24ug (24), 0.35ug (38), 0.4ug (44), 0.5ug (35), 1.5ug (24,25), 1.75ug (38), 2ug (28,41), 2.6ug (40), 3ug (45), 6ug (24,25,36), 20ug (37), 24ug (46), 200ng (31,33), 750ng (34), 200ng/100ul (43), 1ug/500ul (30), 5ug/ul (39), 75ng/mm^2 (27), 2.1ug/mm^3 (32), 0.24ug/mm^3 (29), 1ug/mg (26) and two studies did not mention dosage (23,42).

Studies in favour of using VEGF

The results based on new bone compared to the control were as follows. Eleven articles had a group, time point or dosage with significantly better results in the VEGF group compared to the control. Seven articles showed better bone growth at all time points and dosages for the VEGF group (23,32–35,40,41). One article only showed significantly better results when compared to empty control and not an empty carrier (26). One article favoured VEGF at 8 and 12 weeks (42). One article favoured VEGF at week two, four, and eight (38), and another article favoured VEGF at a dosage of 1.5ug VEGF at week four (24). Of these 11 articles, 6 had empty scaffold as the control group (23,24,33,34,40,41), while four articles had an empty defect as the control group (26,32,38,41) and four compared the combined scaffold and release method without VEGF (35,38,40,42). Evaluations were conducted after an average of 5.8 weeks.

Studies not in favour of using VEGF

In total, 18 articles had a group, time point or dosage with no significant (NS) difference in bone formation in the VEGF groups compared the control group. Twelve articles showed NS compared to control for all groups included in the study (25,26,28–31,36,37,39,43–46). One article showed NS compared to the scaffold (26). One article with the dosage of 0.24ug and 6ug in weeks 4 and 13, and 1.5ug at week 4 (24), while 1 article was NS in the 4-week group (42). One article in weeks 1 and 13 (27) and one in week 12 (38). Of these results, eight had a control of only scaffold (24,25,30,31,36,43–45), two had an empty defect (28,39), two had the scaffold and release method combined (42,44), three had both the empty defect and scaffold group (26,28,38,46) and one had an empty defect and release material (29). The average evaluation was conducted after eight weeks.

One article had a group in week four with significantly lower bone formation in the VEGF group compared to both empty defect and scaffold (27).

The time of evaluation was significantly lower in the 11 articles that showed better results with the use of VEGF compared to the 18 articles showing NS compared to the control ($p<0.05$).

The characteristics of included studies are illustrated in Table 2. The quality scores of the 24 articles range from 3–8 with an average of 5.08 points out of 10. The average quality score of the 11 studies that had a group, time point or dosage with a significantly better bone formation than the control was 4.81, whereas the average of the 18 articles with no significant difference was 5.3. No statistical differences were observed between the groups ($p>0.05$). The only article with a lower quantity of bone formation with the use of VEGF had a score of 7.

Table 2

References	1) Peer review journal	2) Control group	3) Randomi-zation	4) VEGF dose justified	5) Blinding	6) Details on animal model	7) Sample size calculation	8) Comply with ethics	9) No conflict of interest	Quality Score
Amirian 2015 (23)	X	X	-	-	-	X	-	X	-	4
Kenney 2009 (32)	X	X	-	-	-	-	-	X	-	3
Lohse 2015 (24)	X	X	-	X	-	-	-	-	-	3
Çakir-Özkan 2017 (34)	X	X	-	-	-	X	-	X	-	4
L Zhang 2014 (42)	X	X	-	-	X	X	-	X	X	6
Lv 2015 (35)	X	X	X	-	-	-	-	X	-	4
Khojasteh 2017 (43)	X	X	-	-	X	-	-	X	-	4
Moser 2017 (25)	X	X	X	X	X	-	-	X	X	7
W Zhang 2014 (36)	X	X	-	-	-	-	-	X	-	3
W Zhang 2011 (37)	X	X	X	-	-	-	-	X	-	4
Quinlan 2015 (26)	X	X	-	X	X	X	-	X	X	7
Schliephake 2015 (27)	X	X	-	-	X	X	X	X	X	7
Behr 2012 (33)	X	X	-	X	-	X	-	X	X	6
Geuze 2012 (44)	X	X	X	X	-	X	X	X	X	8
Hernández 2012 (38)	X	X	-	-	-	X	-	X	-	4
Kempen 2009 (28)	X	X	-	X	-	X	-	X	-	4
Casap 2008 (39)	X	X	X	-	X	X	-	X	-	6
Patel 2008 (29)	X	X	-	X	X	X	-	X	-	6
Yang 2010 (40)	X	X	-	-	X	X	-	X	-	5
Yonamine 2010 (30)	X	X	-	-	-	-	-	X	-	3
Wu 2012 (41)	X	X	X	-	X	X	-	X	X	7
Schmitt 2013 (46)	X	X	-	X	-	X	-	X	-	5
Du 2015 (45)	X	X	X	-	X	X	-	X	X	7
Das 2016 (31)	X	X	-	X	-	X	X	X	-	5

Table 2: *Modified score of quality from CAMARADES for systemic reviews in experimental animal studies* (22)*: (1) peer-reviewed journal; (2) control group; (3) randomisation; (4)VEGF dose justified; (5) blinding; (6) details on animal model; (7) sample size calculation; (8) compliant with ethics; and (9) no conflicts of interest.*

Discussion

Using reproducible search and inclusion methods, this systematic review collated and assessed recent progress in the solitary use of VEGF for bone formation over the past 10 years alongside any progress made towards clinical usage. A total of 1374 articles were found by the search criteria. Phase one included 70 articles by title and abstract, while phase two excluded 46 articles based on the full-text analysis. Ultimately, a total of 24 articles met the criteria for inclusion. These were quality scored using eight validated questions and one modified for the purpose of this review. Notably, the various models and methodologies used in these studies made statistical comparisons

difficult. However, some very exciting indications could be extracted from the articles, such as the most efficient use of VEGF appearing to occur in defect models with a release of VEGF within the first three weeks, and evaluation studies with an early focus of eight weeks or less exhibiting the improved use of VEGF. For future study designs, this review serves as an inspiration for the modification and improvement of VEGF use for bone formation.

In 2008, a systematic review of the use of VEGF for bone formation was produced for future applications in bone research (21). Based on their findings, this review stated the potential use of VEGF in fracture healing and suggested a focus on future implementation. The most recent study included in this review was from January 2007. To our knowledge, no systematic review has been performed on this matter since. This point highlight the relevance of a follow-up study to evaluate further progress regarding the clinical use of this growth factor in bone surgery to promote more efficient healing.

A statistical comparison of all included studies proved difficult due to the inconsistencies in variables such as administration, model, defect, animal, release and dosage across studies. Moreover, an internal difference noted by Schliephake et al. had one group showing NS results in weeks 1 and 13, but a lower amount of bone in week 4. This illustrates that the use of the same design can yield different results depending on the time of evaluation. The time of observation was between 1–13 weeks across all included studies. Moreover, the majority of the included studies had an evaluation between 4–8 weeks (58%). Comparison according to outcome showed a significantly shorter evaluation period in the studies achieving a better effect of VEGF compared to the control. This could be an indication that the greatest effects of VEGF occur within the early stage of the bone regeneration process.

Although dosages were reported in different units, a general observation was that the lowest reported dosage used in all included studies was 0.2ug (31,33), while the highest was 24ug (46). The two dosages with 0.2ug were reported by Behr et al. (who had significantly better results with VEGF) and by Das et al. (who showed no significant difference). Furthermore, Schmitt et al. used 24ug and noted no significant difference compared to control. In studies where VEGF showed significantly better results in all groups, 2.1ug/mm^3 was used in a mouse intra-femoral implementation (32), 0.5ug was used in a rabbit femoral condyle defect (35), 200ng was used in a mouse skull defect (33), 2ug was used in a rabbit mandible distraction model (41), 750ng was used in a rabbit mandible defect (34) and 2.6ug was used in a rabbit radial diaphysis (40), while a rat cranial defect model used an unknown dosage (23). The only group with a worse effect of VEGF than control used 750ng/cm^2 in a rat tibia head implant (27). Based on these markers, it is not possible to provide a dose-response curve in any of the models. However, it still serves as a marker for future studies working with the same animal and defect model with the use of VEGF—even in combination studies.

In translational medicine, the dosages and release rates are essential to provide sufficient evidence for the clinical applications of products (38). Release rates are defined as burst or continuous release. The release pattern of the growth factors has already been previously studied with a focus on prolonging the effect of VEGF (47). In the use of VEGF on bone formation, the general consensus is that VEGF has the greatest effect when released in the natural systemic peak of 2–3 weeks (48) which, in smaller rodent and humans, is calculated to be at approximately week 1–3 (49,50). Theoretically, VEGF will be concentrated in the inflammatory phase during the first week after a fracture within a defect. However, in the cartilage phase, inhibition has been shown to stimulate the osteogenic lineage (51). These statements would suggest that VEGF had an optimal effect in endochondral ossification and that the release should be stopped before the cartilage phase of the bone formation process. This would imply that the optimal release occurs within the first weeks of a defect. By this assumption, we grouped the included studies into burst release groups, with full release occurring within the first three weeks (23,28,30,31,35,39,40,43) and continuous release occurring for more than three weeks (24–26,37,42). The remaining studies have no information regarding release after three weeks (27,34,38) or have an unknown release profile (29,32,33,36,41,44–46).

In the seven studies that had better bone formation than the control, three had a full release within the first three weeks (23,35,40) while the rest were unknown (32–34,41). The only group worse than control had no information on the release (27). The assumption made by these results supports the existing literature, which suggests that VEGF should be released within the first 3 weeks.

The amount of new bone varied from 0–1% in a mouse ectopic model (25) up to 92% in a mouse calvarial model (33), and up to 96% in a rat mandible distraction model (41). Since the physiological effects of VEGF seem to be best in the endochondral state of healing, it follows that the ectopic designs exhibit low amounts of new bone. The other ectopic design in this review had only 4% of total bone formation (44). These results suggest that VEGF is not very osteogenic outside of defects models and the effectiveness is location-dependent. By this assumption, VEGF has the potential to optimise existing chemokine effects, but not to establish new pathways for enhancement. This could theoretically indicate that a focus towards fracture models would be preferable if the optimal use of VEGF should be established.

In the calvarial mouse model by Behr et al., VEGF had the same effect on bone formation as BMP-2. As such, the authors focused on comparing the dosages of BMP-2 to the clinical dosages that are already used for BMP-2 and could be used for VEGF. This study design focused on the translational purpose and the release method of the collagen sponge that has already been used for clinical studies. This study could serve as a marker for future designs in larger animal models with a dosage of 200ng/mouse. This also seems to correlate to the dosage range for growth factors in a

small animal model. Furthermore, BMP-2 was adjusted from a clinical dosage into an experimental dosage. Notably, being able to translate between different models and areas to use the design for multiple purposes is an important aspect of optimal VEGF use.

Overall, 15 of the studies included in this review have the primary purpose of combining the angiogenic stimulation of VEGF with bone morphogenic protein (BMP) or mesenchymal stem cells (23,24,43,44,46,52,53,25,28,31,34–38), whereas the group with only angiogenic stimulation serves as a secondary objective. The general outcome is that 11 of these studies with a combination treatment using BMP (23,24,52,25,28,31,34–36,44,46) showed superior results to treatment using VEGF alone, as measured by new bone formation. Khojasteh et al. showed superior results with a combination of mesenchymal stem cells. Moreover, Wu et al. noted no difference between combination treatment and VEGF at week two, though the combination treatment was significantly better than VEGF alone at weeks four and eight. This illustrates that the time of evaluation serves as an important factor in the measures of the outcome of bone formation with the use of these growth factors.

The majority of control groups in the included articles are empty defects, scaffolds only, or scaffold in combination with release material (Table 1). The clinical relevance for this approach must be considered limited since our intervention has the purpose of providing the same (or even better) results using existing procedures. However, while this can prove difficult in smaller animal models, it should be considered when translating into larger models to provide comparable results. An example of this is a study by Hernández et al., where the authors used the INFUSE model for the delivery of BMP-2—a method that is already established clinically and thus serves as a relevant control group.

In general, the use of VEGF in bone formation studies has shown strong potential over the last decade; however, no human trials have been performed to date. In order to evolve in the field, more descriptive methodologies would be particularly useful so that each study could be compared with existing results and developed to influence future designs. This would provide an opportunity to compare existing results using statistics, thereby creating the transparency necessary to build on existing literature. Notably, this field is currently delayed by the lack of reproducibility.

Conclusion

This review determined that the development of VEGF is still progressing for the purpose of clinical application. The studies included in this systematic review provide valuable information on the use of VEGF for bone formation, such as the release of VEGF appearing to be optimal within the first three weeks following fracture, and studies with evaluations before eight weeks seeming to have the best outcome. The effect on bone formation seems to be higher in defect models when compared to ectopic designs. The dosages in these types of studies are generally lower than 24 ng

per animal considering a variety of models and animals. Finally, this study provides an overview of the methodology for future model designs.

Studies that combined VEGF with other osteogenic factors generally had a higher percentage of new bone; however, this is also correlated to possible higher costs, more preparation, and side effect profiles.

Future research using VEGF alone should be inspired by the existing literature and focus on the development of published methodologies and results. This will ensure progression in the area while making it possible to translate the results of different models and animals for clinical trials.

Conflict of interest

Authors declare no conflicts of interest

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Table 2

Reference	Animal	Type	Scaffold	Control	Type of VEGF	Release	Dosage	TOE	Results	BV/TV	Risk of bias
Amirian 2015 (23)	Rat	Cranial defect model	Pectin-biphasic calcium phosphate	Empty defect	rhVEGF	Gelatin hydrogel scaffolds	Total concentration VEGF: Unknown 75% released in 15 days	2 weeks / 4 weeks	MicroCT: VEGF significantly better than control No mentioned P-values.	MicroCT: Control: 2w/4w 2.3%/3.3% VEGF: 2w/4w 6.4%/6.5% Histomorphometry: Control: 2w/4w 2.13%/3.75% VEGF: 2w/4w 6.74%/8.95%	4
Keeney 2010 (32)	Mice	Intra-femoral defect	Collagen/ calcium phosphate	Only scaffold	Therapeutic plasmid VEGF 165	Plasmid DNA	0.35ug/ mm3	30 days	Significant more bone in scaffold +	Histomorphometry: Control: 9.8%	3

									VEGF165	VEGF (non-complexed): 24.2% VEGF (complexed): 17.1%	
Lohse 2015 (24)	Rat	Mandible	calcium carbonate granules	Scaffold and empty defect	rhVEGF165	poly-DL-lactic acid (PDLLA)	0.24ug/ 1.5ug /6ug. ≈50% release first 3 days, low constant release till 5 weeka.	4 weeks / 13 weeks	Only significantly better with 1.5ug after 4 weeks. NS other timepoint and dosages	Histomorphometry: 4 weeks: 4.4%-7.3% 13 weeks: 2.7%-4.6% Blank scaffold and empty defect, 4 or 13 weeks: 0.6-1.8%	3
Çakır-Özkan 2017 (34)	Rabbit	Mandible	PLLA-PEG	Only scaffold	rhVEGF165	Gelatin	750ng/scaffold. Release 60% first 2 days, constant release for more than 14 days.	4 weeks / 8 weeks	Significantly better than control.	Histomorphometry: Newly formed bone Control: 4w/8w 34%/17% VEGF: 4w/8w 53%/31%	4
L Zhang 2014 (42)	Beagle	Femoral neck fracture model	Cannulated (titanium) screws	Cannulated screw fibrin glue	VEGF	PLGA/Fibrin glue	Unknown total VEGF in fibrin glue. 21.5% released after 3 days. Steady with 1.71% till 42 days with 88% cumulative release.	4 weeks / 8 weeks / 12 weeks	VEGF had significant better results in week 8 and week 12 ($p<0.01$)	4 weeks: Control 5,7% VEGF 8.0% 8 weeks: Control: 19.5% VEGF: 29.0% 12 weeks: Control: 32.3% VEGF 41.3%	6

Lv 2015 (35)	Rabbit	Femoral condyle defect model	Titanium scaffold or empty defect	Empty titanium scaffold	rhVEGF165	Fibrin glue	0.5ug VEGF. Steady 100% release in 96 hours 0.6%/hour steadily from 12 to 96 hours	4 weeks	Significantly better than control	New bone: Control: 7.8-8.3% VEGF: 17.4%	4
Khojasteh 2017 (43)	Dog (mongrel)	Mandible defect	B-TCP	Scaffold only	VEGF	PLGA microspheres	Release: Burst 60ng/ml 8h. Steady 14 days release total 200ng/ml.	8 weeks	No significant difference to control	Histomorphometry_ Control: 7.2% VEGF: 20%	4
Moser 2018 (25)	Rat	Ectopic	PDLLA/CaCO3 composite granules	Scaffold + granules	rhVEGF165	PDLLA/CaCO3	25ug VEGF/g polymer Total dose: 1.5ug VEGF Burst release: 3 days 5.5%/6% total release in 29 days 100ug/g polymer / 6ug VEGF Burst release: 3 days 6% total release in 29 days 10.5%	4 seeks / 13 weeks	No significant difference to control	Histomorphometry: 4 weeks: Control: 0-1% VEGF: 0-1% 13 weeks: Control: 0-1% VEGF: 0-1%	7
W Zhang 2014 (36)	Rabbit	Skull defect	Silk scaffold	Silk scaffold with water	VEGF	Silk scaffold +water absorption	6ug/scaffold	12 weeks	No significant difference to control	uCT BV/TV control: 4.12 VEGF: 10.14 Histomorphometry:	3

										Control: ~28% VEGF ~44%	
W Zhang 2011 (37)	Rabbit	Sinus floor elevation surgery	Silk hydrogel	Silk gel alone	rhVEGF165	Silk hydrogel	1000ug/ml * 0.200 ml = 4 ug per scaffold No burst release. At least 24 days release	4 weeks / 12 weeks	No signficant difference between VEGF and control	Histomorphometry 4 weeks: Control: 1.8% VEGF: 5.6% 12 weeks: Control 8.7% VEGF: 18.5%	4
Quinlan 2017 (26)	Rat	Calvarial defect model	Collagen-Hydroxyapatite scaffold	Empty defect and only scaffold	rhVEGF165	Alginate micropa rticles	1ug/mg (1.6 ug/scaffold) Burst release till day 7. Steady release till 8 weeks.	8 weeks	Significantly better than empty defect. NS against scaffold alone. Significant more new bone in VEGF group	uCT: Empty defect: 0.4% Empty scaffold: 1.8% VEGF: 3.2% Histomorphometry: (um2, 10^5) Empty defect: 0.9 um2 x 10^5 Empty scaffold: 1.9 um2 x 10^5 VEGF: 5.4 um2 x 10^5	7
Schliephake 2015 (27)	Rat	Tibia head placement	Titanium implant	Empty implants Empty implants with empty DNA nucleotide surface	rhVEGF165	DNA oligonucleotide	750ng/screw 53% released within week 1.	1 week / 4 weeks / 13 weeks	Significant lower bone formation in week 4. NS in week 1 and 13.	Histomorphometry: 1 week: Empty control: 4.0% Surface control: 4.3% VEGF: 5.9% 4 weeks Empty control: 17.6% Surface control: 20.3%	7

										VEGF: 5.9% 13 weeks No values mentioned	
Behr 2012 (33)	Mouse	Calvarial model	Collagen sponge	PBS soaked collagen sponges	VEGF A	Collagen sponge	200ng/ mouse	2 weeks / 4 weeks / 8 weeks / 12 weeks	VEGF significantly better than control.	uCT 2 weeks: Control:1.2% VEGF:81.0% 4 weeks: Control:0-2% VEGF: ~95% 8 weeks: Control:~12% VEGF: ~90% 12 weeks: Control: 18.3% VEGF: 95.1% Histomorphometry: 3 weeks: Control: ~2% VEGF: ~55% 12 weeks: Control: ~11% VEGF: ~65%	6
Geuze 2012 (44)	Beagle	Ectopic	BCP scaffold	Calcium phosphate BCP scaffold mixed with microparticles or hydrogel without growth factors	rhVEGF165	Sustained release: PLGA microparticles. Fast release: Hydrogel (gelatin)	0.4ug per ectopic implant	9 weeks	No significant difference to control.	Histomorphometry: PLGA release: Control:0-1% VEGF:0-1% Hydrogel release: Control: 0-% VEGF: 3-4%	8
Hernandez 2012 (38)	Rabbit	Bone defect condyle femur	PLGA porous scaffold	Empty defect and empty	rhVEGF165	PLGA microspheres	4mg (0.35ug) / 20 mg (1.75ug)	2 weeks / 4 we	2 weeks: VEGF (1.75) signific	4 weeks Control: 10% VEGF: 20%	4

			scaffold			50% release after 4 days. Around 90% in 2 weeks (fig says 2 weeks, text 3 weeks fig. 3a)	eks / 8 weeks / 12 weeks	antly better than all groups 4 weeks: significantly better in VEGF group. 8 weeks: no difference from week 4. Data not shown. 12 weeks: no difference in control and VEGF.	12 weeks Control: 10-15% VEGF: 18-20%	
Kempen 2009 (28)	Rat	Critical sized femur shaft model, subcutaneous model	Empty defects (only orthotopic) and empty scaffold	VEGF	Gelatine Hydrogel	2.0ug/scaffold 58% release during first 3.5 days. Total release after 2 weeks	8 weeks	Subcutaneous: No significant difference between control and VEGF. Orthotopic: No significant difference between VEGF and empty scaffold or	Subcutaneous: Empty scaffold: 0% VEGF: 0% Orthotopic¢: uCT new bone; Empty defect: 28mm3 Empty scaffold: 28mm3 VEGF: 30mm3	4

									empty defect.		
Casap 2008 (39)	Rabbit	Mandible distraction	Injections	No injection	rVEGF165	-	After 14 days 5ug/uL for 4 days.	60 days	No significant difference to control (p=0.057)	MicroCT BV/TV: Control: 2.5% VEGF: 13%	6
Patel 2008 (29)	rat	Cranial defect	Gelatin microspheres in porous PPF scaffold	Blank Gelatin microspheres or empty defect	VEGF	Gelatin	0.24ug/mm^3	4 weeks / 12 weeks	No significant difference to control	Histomorphometry scoring: Control 4 weeks: 0.5 12 weeks: 1 VEGF 4 weeks: 0.5 12 weeks: 1 MicroCT Empty defect: 4w: 7% 12w: 16% Control 4 weeks: 4% 12 weeks: 8% VEGF 4 weeks: 2% 12 weeks: 6%	6
Yang 2009 (40)	Rabbit	Radial diaphysis	BTCP coated with fibrin sealant	Scaffold and untreated	rhVEGF165	Absorption fibrinogen	2.6ug VEGF/scaffold 90% release after 7 days.	4 weeks, 8 weeks, 12 weeks	Significant difference to control	microCT new bone/TV 4 weeks: Control: 18% VEGF:33 % 8 weeks: Control:34 % VEGF: 63% 12 weeks:	5

										Control: 55% VEGF: 18%	
Yonamine 2010 (30)	Rat	calvaria	PLGA microspheres	Empty defect / sham surgery	VEGF 165	PLGA microspheres	1ug per 500ul No release from day 0-7. Full release till day 21.	12 weeks	No significant difference to control	X-ray: Control: 20% VEGF:27-28%	3
Wu 2012 (41)	rabbit	Mandibular distraction	Plasmid pIRES injection	pIRES and normal saline	hVEGF165	Plasmid	2ug	2 weeks / 4 weeks / 8 weeks	Significant difference to control in both bone types.	Histomorphometry: Cortical 2 weeks: Saline:33 % pIRES: 35% VEGF: 39% 4 weeks: Saline:81 % pIRES: 84% VEGF: 93% 8 weeks: Control:91 % pIRES: 93% VEGF: 96% Trabecular 2 weeks: Saline: 23% pIRES: 23% VEGF: 25% 4 weeks: Saline: 41% pIRES: 43% VEGF: 47% 8 weeks:	7

										Control:43 % pIRES:46 % VEGF: 53%	
Schmitt 2013 (46)	pigs	Calvaria defect or vertical augmentation	Bio-oss	Bio-oss collagen carriers (empty scaffold)	rhVEGF165	Fibrin glue	8ug/ml, 3 ml total	30 days / 60 days	No significant difference to control	X-ray Critical size defect: 30 days: Control: 17% VEGF: 19% Vertical augmentation: 30 days: Control: 6% VEGF 4% 60 days Control: 10% VEGF: 11%	5
Du 2015 (45)	Beagle	Mandible defect	Nano-hydroxyapatite coral blocks	Only scaffold	rhVEGF165	Absorption to scaffold	3ug per scaffold block	3 weeks / 8 weeks	No significant difference to control	Histomorphometry 3 weeks: Control: 22% VEGF: 27% 8 weeks: Control: 33% VEGF: 39%	7
Das 2016 (31)	Rats	Mandible	PLGA microsphere	Empty defect	rhVEGF	PLGA microsphere	200ng. steady release 3 weeks.	12 weeks	No significant difference to control	Histomorphometry 12 weeks: Control: 1% VEGF 5%	5

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