	Inventaris Wob-verzoek W16-13S								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2016445								
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel			x					
3	Niet-technische samenvatting	x							
4	Bijlage beschrijving dierproeven 1 oud			x					
5	Bijlage beschrijving dierproeven 2 oud			x					
6	DEC-advies			x					
7	Ontvangstbevestiging				x		x	x	
8	Mail verzoek aanvulling				x		x	x	
9	Reactie verzoek aanvulling				x		x	x	
10	Bijlage beschrijving dierproeven 1 herzien			x					
11	Bijlage beschrijving dierproeven 2 herzien			x					
12	Advies CCD		x						x
13	Beschikking en vergunning				x		x	x	
14	Mail terugkoppeling DEC 19-4-2016				x		x	x	

29 FEB 2016

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.zbo-ccd.nl of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☑ Ja > *Vul uw deelnemernummer in*	11500
☐ Nee > *U kunt geen aanvraag doen*	

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	UMC Utrecht
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	3 0 2 4 4 1 9 7

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

Straat en huisnummer	Instantie voor Dierenwelzijn
Postbus	12007
Postcode en plaats	3501AA Utrecht
IBAN	NL27INGB0000425267
Tenaamstelling van het rekeningnummer	Universiteit Utrecht

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☑ Dhr. ☐ Mw.
Functie	PhD onderzoeker
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☑ Dhr. ☐ Mw.
Functie	[redacted]
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	[redacted] ☐ Dhr. ☑ Mw.
		Functie	[redacted]
		Afdeling	[redacted]
		Telefoonnummer	[redacted]
		E-mailadres	[redacted]
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☑ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn
		Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn
		Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier
		☐ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☐ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	01-01-2016
		Einddatum	01-01-2021
3.2	Wat is de titel van het project?	Smart coatings for orthopedic implants	
3.3	Wat is de titel van de niet-technische samenvatting?	Slimme coatings voor orthopedische implantaten	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC Utrecht
		Postadres	Postbus 85500 3508 GA Utrecht
		E-mailadres	dec-utrecht@umcutrecht.nl

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

- [x] Nieuwe aanvraag Projectvergunning € 1.187,00 Lege
- [ ] Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

- [ ] Via een eenmalige incasso
- [x] Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- [x] Projectvoorstel
- [x] Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- [ ] Melding Machtiging
- [x] Bijlage dierproef 1, bijlage dierproef 2

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.6). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	[redacted]
Plaats	Utrecht
Datum	19-12-2016
Handtekening	[redacted]

[redacted]

2

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500
1.2	Provide the name of the licenced establishment.	UMC Utrecht
1.3	Provide the title of the project.	Smart coatings for orthopedic implants

2 Categories

2.1	Please tick each of the following boxes that applies to your project.	☒ Basic research
		☒ Translational or applied research
		☐ Regulatory use or routine production
		☐ Research into environmental protection in the interest of human or animal health or welfare
		☐ Research aimed at preserving the species subjected to procedures
		☐ Higher education or training
		☐ Forensic enquiries

☐ Maintenance of colonies of genetically altered animals not used in other animal

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Although several kinds of implants are approved for their use in orthopedic surgeries, failure still occurs in 10% of the cases [1] mainly due to 1) deficient integration of the implant with the surrounding bone and due to 2) implant-associated infections. These processes are highly interdependent. The development of simple and stable implants having optimal bone-stimulating and bacterial-killing properties is therefore of scientific and clinical significance [1, 2].

According to clinical observations, an implant may provide a surface at which bacteria escape immune surveillance. As such, bacteria can form a biofilm (i.e. a combination of bacteria and secreted polymers) on the implant surface, which subsequently protects them from clearance by the immune system and treatment with antibiotics [3]. This results in a chronic infection of the implant. The infection also impairs the adhesion of bone cells, thus reducing the bone-implant integration. The current treatment of patients with antibiotics furthermore has some limitations, such as local tissue damage [3] and the risk of antibiotic resistance [4].

In our in vitro research, we design and test coatings with bone-stimulating and bacterial-killing properties. For this purpose, we use complex surface treatments of materials (e.g. nanotubes) which can be loaded with inorganic particles (e.g. silver or zinc) [5,6,7]. Furthermore, bone-stimulating factors such as bone morphogenetic proteins or inflammation-associated factors can be incorporated in the coatings [8,9]. As part of the latter, we have shown that bacterial-derived factors (e.g. proteins, lipoproteins or teichoic acids) have bone-stimulating properties [10, 11]. To test whether these coatings can indeed prevent biofilm formation and improve bone integration, this study aims to test the efficacy of different coatings in the context of the immune system in animal models. Both metal and calcium phosphate ceramic implants will be tested, as they are both interesting materials for orthopedic applications.

Recently, there has been a huge progress in the fabrication methods of porous implants, for instance by the advances in additive manufacturing techniques [1,2,12-15]. This has opened up new paths to tackle implant-associated infections by making well-designed implant coatings with precise architecture. For metal implants, titanium nanotubes (NTs) coatings have been demonstrated to promote adhesion and matrix production by bone cells *in vitro* [16] and bone-implant integration *in vivo*. In addition, these NTs constitute an excellent drug-loading and delivering platform [17,18,19]. Using the NTs, the loading capacity and the release rate of the drugs can be easily varied by changing the structural parameters. The NTs can be further modified in a number of ways to enhance their bacterial-killing performance. First, to further fine-tune the delivery of incorporated inorganic nanoparticles (e.g. zinc, silver) from the coatings, polymeric hydrogels can act as a reservoir in the NTs for their sustained release [20, 21]. Second, dual-purpose implants – i.e. implants that enhance stimulate bone formation and kill

bacteria- have recently gained more interest [12,13]. We hypothesize that the combination of a bacterial-killing agent with a bone-stimulating growth factor would enable us to control the dose and the timing of these components for a longer period [22,23]. We will include various bone-stimulating growth factors (e.g. bone morphogenetic proteins) and inflammation-associated compounds (e.g. proinflammatory cytokines or bacterial-derived factors) [8,9,10,11] known as beneficial for bone formation. We would therefore like to test if the coating of materials with these compounds is a clinically feasible strategy to improve bone formation. These biomolecule coatings can not only improve the integration of implants with surrounding bone, but they may also be used in many other bone regeneration applications as a replacement of the current bone grafts [24].

REFERENCES

[1] Amin Yavari S, van der Stok J, Chai YC, Wauthle R, Tahmasebi Birgani Z, Habibovic P, et al. Bone regeneration performance of surface-treated porous titanium. Biomaterials 2014;35:6172-81.
[2] Amin Yavari S, Chai YC, Böttger AJ, Wauthle R, Schrooten J, Weinans H, et al. Effects of anodizing parameters and heat treatment on nanotopographical features, bioactivity, and cell culture response of additively manufactured porous titanium. Materials Science and Engineering: C 2015;51:132-8.
[3]Hickok NJ, Shapiro IM. Immobilized antibiotics to prevent orthopaedic implant infections. Advanced Drug Delivery Reviews 2012;64:1165-76.
[4] Costerton JW. Biofilm theory can guide the treatment of device-related orthopaedic infections. Clinical Orthopaedics and Related Research 2005:7-11.
[5] Jin G, Qin H, Cao H, Qiao Y, Zhao Y, Peng X, et al. Zn/Ag micro-galvanic couples formed on titanium and osseointegration effects in the presence of S. aureus. Biomaterials 2015;65:22-31.
[6] Necula BS, Fratila-Apachitei LE, Zaat SAJ, Apachitei I, Duszczyk J. In vitro antibacterial activity of porous TiO2–Ag composite layers against methicillin-resistant Staphylococcus aureus. Acta Biomaterialia 2009;5:3573-80.
[7] Qin H, Cao H, Zhao Y, Zhu C, Cheng T, Wang Q, et al. In vitro and in vivo anti-biofilm effects of silver nanoparticles immobilized on titanium. Biomaterials 2014;35:9114-25
[8] Yun YR, Jang JH, Jeon E, Kang W, Lee S, Won JE, Kim HW, Wall I. Administration of growth factors for bone regeneration. Regen Med. 2012 May;7(3):369-85.
[9] Mountziaris PM, Spicer PP, Kasper FK, Mikos ASTissue Eng Part B Rev. Harnessing and modulating inflammation in strategies for bone regeneration. 2011 Dec;17(6):393-402
[10] Croes M, Oner FC, Kruyt MC, Blokhuis TJ, Bastian O, Dhert WJ, Alblas J. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. PLoS One. 2015 Jul 15
[11] Croes M, Loozen L, Kruyt MC, Yuan H, Dhert WJ, Oner FC, Alblas J, Local inflammation and bone formation in a new translational animal model. Unpublished.
[12] Amin Yavari S, Wauthle R, van der Stok J, Riemslag AC, Janssen M, Mulier M, et al. Fatigue behavior of porous biomaterials manufactured using selective laser melting. Materials Science and Engineering: C 2013;33:4849-58.
[13] Wauthle R, Van der Stok J, Yavari SA, Van Humbeeck J, Kruth J-P, Zadpoor AA, et al. Additively manufactured porous tantalum implants. Acta Biomaterialia 2014.
[14] Ahmadi SM, Campoli G, Amin Yavari S, Sajadi B, Wauthle R, Schrooten J, et al. Mechanical behavior of regular open-cell porous biomaterials made of diamond lattice unit cells. Journal of the Mechanical Behavior of Biomedical Materials 2014;34:106-15.
[15] Amin Yavari S, Ahmadi SM, van der Stok J, Wauthle R, Riemslag AC, Janssen M, et al. Effects of bio-functionalizing surface treatments on the mechanical behavior of open porous titanium biomaterials. Journal of the Mechanical Behavior of Biomedical Materials 2014;36:109-19.

[16] Popat KC, Leoni L, Grimes CA, Desai TA. Influence of engineered titania nanotubular surfaces on bone cells. Biomaterials 2007;28:3188-97.
[17] Gao A, Hang R, Huang X, Zhao L, Zhang X, Wang L, et al. The effects of titania nanotubes with embedded silver oxide nanoparticles on bacteria and osteoblasts. Biomaterials 2014;35:4223-35.
[18] Mei S, Wang H, Wang W, Tong L, Pan H, Ruan C, et al. Antibacterial effects and biocompatibility of titanium surfaces with graded silver incorporation in titania nanotubes. Biomaterials 2014;35:4255-65.
[19] Jin G, Qin H, Cao H, Qiao Y, Zhao Y, Peng X, et al. Zn/Ag micro-galvanic couples formed on titanium and osseointegration effects in the presence of S. aureus. Biomaterials 2015;65:22-31.
[20] Nair LS, Laurencin CT. Polymers as biomaterials for tissue engineering and controlled drug delivery. Tissue engineering I: Springer; 2006. p. 47-90.
[21] Kretlow JD, Young S, Klouda L, Wong M, Mikos AG. Injectable biomaterials for regenerating complex craniofacial tissues. Advanced Materials 2009;21:3368-93.
[22] Wenke JC, Guelcher SA. Dual delivery of an antibiotic and a growth factor addresses both the microbiological and biological challenges of contaminated bone fractures. Expert Opinion on Drug Delivery 2011;8:1555-69.
[23] Guelcher SA, Brown KV, Li B, Guda T, Lee B-H, Wenke JC. Dual-purpose bone grafts improve healing and reduce infection. Journal of orthopaedic trauma 2011;25:477-82.
[24] Gupta A, Kukkar N, Sharif K, Main BJ, Albers CE, El-Amin Iii SF. Bone graft substitutes for spine fusion: A brief review. World J Orthop. 2015 Jul 18;6(6):449-56

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The goal of this project is to test multifunctional implant coatings which are designed to facilitate bone integration and bacterial-killing. These coatings are developed and tested as part of another in vitro project.

As a first subgoal, we would like to determine if an implant coating consisting of inorganic nanoparticles (e.g. silver and zinc) in combination with nanotubes is a clinically feasible strategy for bone-implant integration and bacterial-killing purposes. In these study, the effect of the release-rate of these inorganic nanoparticles will also be assessed. The inorganic nanoparticles can be combined with bone-stimulating growth factors (e.g. bone morphogenetic proteins) to enhance the integration of the implants with existing bone.

As a second subgoal, we would like to test whether the coating of materials (i.e. metals or calcium posphates) with inflammation-associated factors (e.g. proinflammatory cytokines or bacterial-derived compounds) is a clinically feasible strategy to enhance their bone-stimulating properties. The coatings can be used to induce bone regeneration as part of a bone substitute or to improve the implant-bone integration of

implants.

We believe our objective is achievable, as we have already identified a number of bacterial-killing and bone-stimulating factors *in vitro* [1,2,3,4]. However, a 5 year time period seems to be realistic, as some manufacturing techniques (i.e. the layer-by-layer release system and hydrogel coating method) have to be optimized and tested in vitro. We believe we have a strong interdisciplinary team of researchers: engineers working on the complex coatings (collaboration with TU Delft), biologists working on stem cells and bone regeneration, and clinicians with knowledge of the translational aspects. Furthermore, our department has experience with the animal models described in this proposal and the required analyses methods. For the rat infection model, we will perform a pilot study to first determine the minimal inoculation dose of *Staphylococcus aureus*. For the rabbit infection model, these dose response studies have already been performed within the same experimental conditions (location, type of animals, origin of animals, and bacterial strain). The animals models used in this project (implant infection model and spinal fusion model) are well described in the literature [5,6] .

REFERENCES

[1]Amin Yavari S, Ahmadi SM, van der Stok J, Wauthle R, Riemslag AC, Janssen M, et al. Effects of bio-functionalizing surface treatments on the mechanical behavior of open porous titanium biomaterials. Journal of the Mechanical Behavior of Biomedical Materials 2014;36:109-19.
[2] Popat KC, Leoni L, Grimes CA, Desai TA. Influence of engineered titania nanotubular surfaces on bone cells. Biomaterials 2007;28:3188-97.
[3] Croes M, Oner FC, Kruyt MC, Blokhuis TJ, Bastian O, Dhert WJ, Alblas J. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. PLoS One. 2015 Jul 15
[4] Croes M, Loozen L, Kruyt MC, Yuan H, Dhert WJ, Oner FC, Alblas J, Local inflammation and bone formation in a new translational animal model. Unpublished.
[5] Reizner, JG Hunter, NT O'Malley, RD Southgate, EM Schwarz, SL Kates. A systematic review of animal models for Staphylococcus aureus osteomyelitis. Eur Cell Mater. 2014 Mar 25;27:196-212.
[6] Drespe et al. Animal models for spinal fusion. Spine J. 2005.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

One of the most important challenges that orthopedic surgeons face, is to minimize infection of implants during and after surgery, while stimulating the integration of the implant with the bone. In 10% of the cases however, implant failure still occurs [1]. It is thought that the prevention or successful treatment of implant-related infections can help hundreds of thousands of patients each year. These patients face revision surgery due to implant loosening and/or implant infection, causing much pain, disability or even death. Apart from this, costly revision surgeries constitute an economical burden on society.

Furthermore, growth factors such as the bone morphogenetic proteins [2], proinflammatory cytokines [3] or and bacterial-derived compounds [4,5] can stimulate new bone formation and therefore may be used as a coating to improve the bone formation around implants. Similarly, these same factors could be used in coatings to stimulate the bone formation around metal or ceramic implants in regenerative strategies (e.g. spinal fusion or non-healing fractures) [6]. Considering that bone is the most transplanted tissue after the transfusion of blood, there is

a great need for new bone substitutes [7].

We hypothesize that a smart implant coating could prevent bacterial colonization, enhance bone integration and stimulate bone regeneration.

The animal studies can also provide us new scientific knowledge: the in vivo performance of these coatings can provide information on the ability of bacteria to produce biofilms on different surfaces. Furthermore, it can elucidate how bacteria can negatively affect the bone tissue. If bone-stimulating compounds are identified, this can give insight in the pathways of bone formation that should be targeted in future research.

REFERENCES

[1] Amin Yavari S, van der Stok J, Chai YC, Wauthle R, Tahmasebi Birgani Z, Habibovic P, et al. Bone regeneration performance of surface-treated porous titanium. Biomaterials 2014;35:6172-81.
[2] Yun YR, Jang JH, Jeon E, Kang W, Lee S, Won JE, Kim HW, Wall I. Administration of growth factors for bone regeneration. Regen Med. 2012 May;7(3):369-85.
[3] Mountziaris PM, Spicer PP, Kasper FK, Mikos ASTissue Eng Part B Rev. Harnessing and modulating inflammation in strategies for bone regeneration. 2011 Dec;17(6):393-402
[4] Croes M, Oner FC, Kruyt MC, Blokhuis TJ, Bastian O, Dhert WJ, Alblas J. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. PLoS One. 2015 Jul 15
[5] Croes M, Loozen L, Kruyt MC, Yuan H, Dhert WJ, Oner FC, Alblas J, Local inflammation and bone formation in a new translational animal model. Unpublished.
[6] US Centers for Disease Control and Prevention: ttp://www.cdc.gov/ncidod/dhqp/tissueTransplantsFAQ.html#d
[7] Giannoudis PV, Dinopoulos H, Tsiridis E: Bone substitutes: An update. Injury 2005Nov;36: S20-S27.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

As part of another project, in vitro antibacterial and pro-osteogenic screening studies will be employed to find optimal dose and time co-delivery of inorganic nanoparticles (e.g. silver or zinc elements) or biological factors (e.g. bone morphogenetic proteins, proinflammatory cytokines or bacterial-derived factors) that can eradicate bacterial colonization and biofilm formation while stimulating the necessary bone formation for bone-implant integration.

Although bacterial killing can be studied in vitro, this does not always correlate with the clinical situation. Therefore, as part of the current project, our strategies should also be tested in the context of the immune system in animal models [1,2,3]. Similarly, we can identify possible bone-stimulating factors in vitro by studying their potential to differentiate stem cells into bone cells. However, as bone cannot be formed in an in vitro setting, the true bone-stimulating potential of our strategies should be tested in animal models.

An implant-related infection model is useful to demonstrate the efficacy of the material coatings in terms of bacterial killing and bone-implant integration [3]. We will induce an infection with *Staphylococcus aureus* (*S. aureus*) and insert a titanium implant to support colonization by these bacteria. The rat implant-infection model allows for more mechanistic studies, while the rabbit implant infection model allows for more clinically representative bacterial doses to be used together with the study of local and systemic signs of inflammation [4].

In contrary to the implant infection model, the spinal fusion model is particularly useful to demonstrate the efficacy of the material coatings as part of bone substitutes in terms of new bone formation [5]. As spinal fusion is the most common application for which bone grafts are used [6], it is logical to test the coatings in the same location from a translational point of view. This model is a better model to study de novo bone formation, whereas the implant model is a better model to study bone growth towards the material.

For both models, we would like to use rats and rabbits. Studies are first performed in rats, as there are more research tools (e.g. antibodies) available for the rat species allowing for more mechanistic studies. Subsequently, findings will be confirmed in rabbits. Literature suggests that the rabbit osteomyelitis model is superior than the rat model for translational research as their immune system is more comparable to that of humans [4,7,8]. Also,in the spinal fusion model, a direct comparison can be made between our coating and the golden standard (i.e. autologous bone grafting). Finally, slightly larger constructs can be tested in rabbits with more freedom of surface modifications.

REFERENCES

[1] McCabe, W. R., and T. L. Treadwell. 1986. In vitro susceptibility tests: correlations between sensitivity testing and clinical outcome in infected patients, p. 925-936. In V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams and Wilkins Co., Baltimore.
[2] Curtis, F. R., and A. E. Wildnson. 1958. A comparison of the in vitro sensitivity of gonococci to penicillin with the results of treatment. Br. J. Vener. Dis. 34:70-78.
[3] Gerber, A. U., and W. A. Craig. 1981. Worldwide clinical experience with cefoperazone. Drugs 22(Suppl. 1):108-118.
[4] Reizner, JG Hunter, NT O'Malley, RD Southgate, EM Schwarz, SL Kates. A systematic review of animal models for *Staphylococcus aureus* osteomyelitis. Eur Cell Mater. 2014 Mar 25;27:196-212.
[5] AM Riordan, R Rangarajan, JW Balts, WK Hsu, PA Anderson. Reliability of the Rabbit Postero-Lateral Spinal Fusion Model: A Meta-Analysis. JOR 2013
[6] WG De Long, TA Einhorn, K Koval et al. Current concepts review: Bone grafts and bone graft substitutes in orthopaedic trauma surgery. J Bone Joint Surg AM 2007
[7] Bosze, Z. and Houdebine, L.M. (2006) Application of rabbits in biomedical research: a review. World Rabbit Sci. 14:1-14.
[8] Dharmadhikari, A. and Nardell, E.A. (2008) What animal models teach humans about tuberculosis. Am. J. Respir. Cell Mol. Biol. 39:503-508.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

We intend to use rat and rabbit models of bone formation and implant-related infection. We will test the efficacy of a number of coatings which are developed and studied as part of another in vitro project.

In the model of implant-related infection, an implant is placed in the tibial cavity after inoculation of *S. aureus*. At a certain inoculation dosage, biofilm formation and chronic infection can be established. Both rat and rabbit models are well-validated for this purpose [1]. In our studies, we will monitor bacterial killing (bacterial survival/proliferation) and implant-bone integration (micro-CT imaging and histology). The inoculation dose in rats will be determined first in a pilot study. In an identical model in rabbits, an inoculation dose of 10^5 CFU with *S. aureus* has already been established to be optimal by our group (unpublished data).

Initial studies will be performed in rats. In these studies, we will assess:
1) different antimicrobial inorganic nanoparticles (including zinc and silver) in combination with nanotubes
2) feasibility of controlling the release-rate of the nanoparticles by adding a hydrogel layer in the nanotubes
3) testing the efficacy of a layer-by-layer design of an implant coating to simultaneously stimulate bone formation and bacterial killing

Subsequently, the most optimal conditions can be tested in the similar model in rabbits. In rabbits, large-size implants can be tested. Also, the inoculation dose of *S. aureus* is more representative of the clinical situation in rabbits than is in rats [2]. Finally, local and systemic effects of the implant coatings can be more easily tested in rabbits.

While the implant-related infection models can be used to study their effect both on bacterial killing and bone-implant integration, these are not functional models in which bone-stimulating coatings can be studied for the purpose of bone regeneration. To assess the bone-stimulating properties of biological or inorganic coatings, we intend to use the spinal fusion model as a second animal model [3, 4]. In this model, bone formation can be easily quantified by determining the fusion rate. The spinal fusion model better mimics a clinical situation where an excessive amount of de novo bone formation is required. The rat spinal fusion model allows for more mechanistic studies - due to the available research tools for the rat species - in combination with 3D imaging. For follow-up studies, rabbits are preferred as their immune system is more comparable to humans [5]. As such, mice and rats are relatively resistant to pro-inflammatory factors and bacterial compounds [6]. These factors candidates to be tested for their bone-stimulating properties.

REFERENCES

[1] Ma M, Kazemzadeh-Narbat M, Hui Y, Lu S, Ding C, Chen DDY, et al. Local delivery of antimicrobial peptides using self-organized TiO2 nanotube arrays for peri-implant infections. Journal of Biomedical Materials Research Part A 2012;100A:278-85.
[2]Reizner, JG Hunter, NT O'Malley, RD Southgate, EM Schwarz, SL Kates. A systematic review of animal models for *Staphylococcus aureus* osteomyelitis. Eur Cell Mater. 2014 Mar 25;27:196-212.
[3] SD Boden, GJ Martin, M Morone, JL Ugbo, L Titus, WC Hutton. The use of coralline hydroxyapatite with bone marrow, autogenous bone graft, or osteoinductive bone protein extract for posterolateral lumbar spine fusion. Spine. 1999;24:320–327
[4] IH Drespe et al. Animal models for spinal fusion. Spine J. 2005.
[5] Copeland S, Warren HS, Lowry, SF, Calvano SE, Remick D. Acute Inflammatory Response to Endotoxin in Mice and Humans. Clin Diagn Lab Immunol.
2005 Jan; 12(1): 60–67
[6] Jacquier V, Estellé, Schmaltz-Panneau B et al. Genome-wide immunity studies in the rabbit: transcriptome variations in peripheral blood mononuclear cells after in vitro stimulation by LPS or PMA-Ionomycin. BMC Genomics 2015, 16:26

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

We can define the following phases in our project. The choice of the animal studies is determined by a number of go/no go moments.

Phase I, in rats (implant-related infection model): we will perform a pilot study first to determine the minimal *S. aureus* dose (CFU) necessary to get a reproducible implant-related infection. Currently, *S. aureus* ATCC 6538 is used for in vitro experiments and it is likely that this strain will also be used for the in vivo experiments. The range is based on literature and will be in the 1x10^2 – 1x10^8 CFU range [1].

Phase II, in rats (implant-related infection model): we will test inorganic nanoparticles (e.g. zinc and silver) using this implant infection model (as determined in phase I). Both the optimal delivery method and concentration of these nanoparticles is studied. We are currently in the process of identifying the efficacy of the nanoparticles in vitro.

Phase III, in rats (implant-related infection model): Go/no go moment. We are currently still developing the technology for the layer-by-layer design for the combined delivery of growth facors and bacterial killing compounds in an in vitro setting. The layer-by-layer coatings will only be tested in animals if they show a higher efficacy in vitro compared to single layer coatings. In these animal studies, we would like to test the optimal layer-by-layer design for bacterial killing and implant integration.

Phase IV, in rabbits (implant-related infection model): go/no go moment. Determine the efficacy of the optimal bacterial-killing and bone promoting coatings (as determined in Phases II and III) in rabbits. These studies will only be performed when inorganic nanoparticles (e.g. silver and zinc) or layer-by layer coatings show an efficacy in the rats. In the rabbits, local and systemic markers of inflammation can be studied. Also, slightly larger implants can be used which allow for more freedom in the surface modification which can be performed. For the rabbit infection model, we will use 1x10^5 CFU *S. aureus*. A pilot study has previously been performed within our group (unpublished data). In this study, a range of *S. aureus* doses (10^2 CFU to 10^6) were compared. The 1x10^5 CFU dose resulted in a reproducible, local, chronic infection in the animals. This was shown to be the optimal concentration in previous dose-response studies performed by our group with the same bacteria in the same rabbit model.

Phase V, in rats (spinal fusion model): determine the in vivo efficacy of bone-promoting coatings for bone regenerative purposes in a functional spinal fusion model in rats.

Phase VI, in rabbits (spinal fusion model): go/no go moment. Determine the efficacy of bone-promoting coatings for bone regenerative purposes in a functional spinal fusion model in rabbits (as determined in Phase V). This study will only be performed when beneficial effects of the bone-promoting coatings are observed in the rats. In the rabbit model, a comparison can be made with the golden standard for bone grafting (autologous) bone. In addition, local and systemic inflammation markers will be studied.

REFERENCES

[1] Reizner, JG Hunter, NT O'Malley, RD Southgate, EM Schwarz, SL Kates. A systematic review of animal models for *Staphylococcus aureus* osteomyelitis. Eur Cell Mater. 2014 Mar 25;27:196-212.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Implant infection model in rats and rabbits
2	Spinal fusion model in rats and rabbits in combination with ectopic implants
3	
5	
6	
7	
8	
9	
10	

Bijlage
Beschrijving dierproeven

- Deze bijlage voegt u bij uw projectvoorstel dierproeven.
- Per type dierproef moet u deze bijlage invullen en toevoegen.
- Meer informatie vindt u op de website www.zbo-ccd.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Vul uw deelnemernummer van de NVWA in.	11500
1.2	Vul de naam van de instelling of organisatie in.	UMC Utrecht
1.3	Vul het volgnummer en het type dierproef in. *Gebruik de volgnummers van vraag 3.4.4 van het format Projectvoorstel.*	Volgnummer: 1 Type dierproef: Implant infection model in rats and rabbits

2 Beschrijving dierproeven

A. Experimentele aanpak en primaire uitkomstparameters

Beschrijf de keuze van de experimentele aanpak en de primaire uitkomstparameters.

We would like to use a rat and rabbit model of an implant-related infection [1]. In this model, a metal implant is inserted in the tibiae of the animals while it is in the meantime inoculated with a specific dose of bacteria [2]. We will use S. aureus to induce a chronic infection, as this is a clinically relevant strain which is often associated with biofilm formation [3]. As we are using S. aureus ATCC 6538 for our in vitro studies, we will likely also use this strain for the animal studies. A pilot study will be performed in the rat model to determine the minimal dose of S. aureus needed to induce a chronic infection. For the rabbit model, such a pilot has already been performed within our group. As such, a dose of 10^5 CFU will be used. After several weeks (4 weeks normally, and 8 weeks when long-term effects are studied) [1] , the number of bacteria can be quantified as a measure of bacterial killing (bacterial culture and

histology). Furthermore, bone formation around the implant is quantified to determine the bone-implant integration (micro-CT imaging and histology) [2].

The bacterial-killing activity and bone-stimulating activity of our implant coatings will be comapared to untreated control implant. In our experiments, we would like to test titanium nanotubes [4], inorganic nanoparticles (e.g. zinc and silver) [5, 6], and layer-by-layer coatings [7] for their bacterial-killing and bone-stimulating activity. Growth factors such as the bone morphogenetic proteins can be used together with the inorganic nanoparticles to promote bone formation in the layer-by-layer coatings.

We will perform initial studies in rats, which have shown to be a very suitable animal model for this purpose due to their size allowing implant placement, easy handling, and the establishment of chronic infection with clinically-relevant bacterial strains [1]. When successful coatings are identified, we would also like to test their efficacy in the rabbit model. The rabbit model, due to their more comparable immune system to humans, is superior to the rat model in a translational aspect [8,9]. Lower doses of bacteria can generally be used in rabbits than in rats, which are more comparable to the clinical situation [1]. For the same reason, in addition to bacterial-killing and bone-implant integration, the rabbit model is a superior model to study local and systemic markers of inflammation. Finally, larger-size implants can be tested which allow for more surface modification.

The different experiments can be summarised af follows:

- test coatings with inorganic nanoparticles (e.g. zinc and silver) in rats. The different elements will be incorporated into titanium nanotube coatings. To embed silver nanoparticles in titanium oxide nanotubes, two different concentrations (0.5 and 1.5 M) of AgNO3 will be used [5]. Also two different concentrations (0.015 and 0.030M) of Zn(NO3)2 will be used [6]. We are currently testing the in vitro bacterial-killing activity of these elements. The animals will be euthanized after 4 weeks.

- test the controlled release of the aforementioned nanoparticles in rats. For this purpose, a thin layer of thermo-sensitive hydrogel will be used in combination with the nanotubes. As we are also interested in the more long-lasting effects of these coatings, animals will be euthanized at two different time points (4 and 8 weeks).

-test a layer-by-layer designed coating with dual purpose, e.g. bacterial-killing and stimulating bone-implant integration in rats. These layers consist of antimicrobials (e.g. antibiotics like gentamicin)[10] and growth factors (e.g. bone morphogenetic proteins)[11]. Here, also two different concentration from each agent will be studied in the same rat model. These components will be tested alone, or in combination, to determine the coating with best bacterial-killing and bone-stimulating activity. We are currently still developing the technology to produce the layer-by-layer coatings. If this is succesful, in vitro release profile of layer-by-layer components will be determined. Depending on the release profile that can be obtained in vitro, the animals will be euthanized after either 4 or 8 weeks.

- test the most promising conditions in the rabbit model. The rabbit model is a more clinically relevant model because lower doses of S. aureus can be used [1]. Furthermore, larger-size implants can be tested which allow for more surface modification. The same analyses will be performed as in the rats. In addition however, blood samples are harvested to measure systemic markers of inflammation. Local signs of inflammation are quantified by histology and cytokine measurements on tissues harvested locally. In this study, the rabbits are euthanized at 4 and 8 weeks to also test the long-lasting effects of the coatings.

REFERENCES

[1] Reizner, JG Hunter, NT O'Malley, RD Southgate, EM Schwarz, SL Kates. A systematic review of animal models for Staphylococcus aureus osteomyelitis. Eur Cell Mater. 2014 Mar 25;27:196-212.
[2] Moojen DJ, Vogely HC, Fleer A, Nikkels PG, Higham PA, Verbout AJ, Castelein RM, Dhert WJ. Prophylaxis of infection and effects on osseointegration using a tobramycin-periapatite coating on titanium implants--an experimental study in the rabbit. J Orthop Res. 2009 Jun;27(6):710-6.
[3] Montanaro L, Speziale P, Campoccia D, Ravaioli S, Cangini I, Pietrocola G, Giannini S, Arciola CR. Scenery of Staphylococcus implant infections in orthopedics. Future Microbiol. 2011 Nov;6(11):1329-49
[4] Gao A, Hang R, Huang X, Zhao L, Zhang X, Wang L, et al. The effects of titania nanotubes with embedded silver oxide nanoparticles on bacteria and osteoblasts. Biomaterials 2014;35:4223-35.
[5] Zhao L, Wang H, Huo K, Cui L, Zhang W, Ni H, et al. Antibacterial nano-structured titania coating incorporated with silver nanoparticles. Biomaterials 2011;32:5706-16.
[6] Liu W, Su P, Chen S, Wang N, Ma Y, Liu Y, et al. Synthesis of TiO2 nanotubes with ZnO nanoparticles to achieve antibacterial properties and stem cell compatibility. Nanoscale 2014;6:9050-62.
[7] Necula BS, Fratila-Apachitei LE, Zaat SAJ, Apachitei I, Duszczyk J. In vitro antibacterial activity of porous TiO2–Ag composite layers against methicillin-resistant Staphylococcus aureus. Acta Biomaterialia 2009;5:3573-80
[8] Bosze, Z. and Houdebine, L.M. (2006) Application of rabbits in biomedical research: a review. World Rabbit Sci. 14:1-14.
[9] Dharmadhikari, A. and Nardell, E.A. (2008) What animal models teach humans about tuberculosis. Am. J. Respir. Cell Mol. Biol. 39:503-508.
[10] Cosgrove SE, Vigliani GA, Fowler VG Jr, Abrutyn E, Corey GR, Levine DP, Rupp ME, Chambers HF, Karchmer AW, Boucher HW. Initial low-dose gentamicin for Staphylococcus aureus bacteremia and endocarditis is nephrotoxic. Clin Infect Dis. 2009 Mar 15;48(6):713-21
[11] Bishop GB, Einhorn TA. Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery. Int Orthop. 2007 Dec; 31(6): 721–727.

Beschrijf de beoogde behandeling van de dieren (inclusief de aard, de frequentie en de duur van de behandelingen waaraan de dieren worden blootgesteld) en onderbouw de gekozen aanpak.

Rats:

Surgical procedure- the left hind leg is shaven and disinfected with povidone iodine. An incision is made to the anteromedial aspect of the tibia. To access the medullary cavity, a hole between the tibial plateau and the tibial tuberosity is drilled with a smooth stainless steel pin (K-wire) through the cancellous bone of the proximal metaphysic. The K-wire is inserted into the medullary cavity and pushed forward distally to create space in the cavity. After removal, a small volume of either saline of bacteria are injected into the medullary cavity by a microsyringe. After bacterial inoculation, the differently coated K-wires are inserted and the skin and fascia are sutured with resorbable sutures. The surgeries are estimated to take half an hour. In vivo scanning is performed with appropriate anesthetics (max. 3 times depending on the duration of the experiment). Fluorochrome markers are injected subcutaneously (max. three time points depending on the duration of the experiment) to determine the onset and development of bone formation. The animals are euthanized after 4 or 8 weeks depending on the coating which is tested.

Anesthetics- start and maintained with isoflurane/oxygen

Pain management-injection analgetics (usually buprenorphine with 12 hour interval), starting before surgery and continued after surgery. The protocol will be developed in consultation with the designated veterinarian. If there is any indication that the rats continue to suffer pain, the pain medication will be prolonged.

Antiseptic techniques- Surgery will be performed under aseptic conditions. The skin will be disinfected with povidone iodine. The surgeon will wear scrubs, sterile gloves, and a surgery mask/cap. Only autoclaved instruments will be used.

Postoperative care- The animals will be placed on heat blankets. The animals will be housed per two if possible. The animals will be scored daily for well-being in the first week, and weekly thereafter. The rats will receive special food if they have reduced food intake due to the surgery and the pain medication.

Infection measurements- In vivo scanning under isoflurane anesthetics is performed for a maximum of 3 times in total. Bacterial culture of bone and implant after euthanization is the primary outcome measure for bacterial killing. Local inflammation markers will be measured on tissue samples.

Bone formation measurements- Bone-implant contact as determined on histology and aforementioned in vivo scans. The incorporation of fluorchromes is assessed by fluorescence microscope. This will elucidate the onset and progression of bone formation around the implant.

Euthanasia protocol- One of the methods listed in appendix IV of directive 2010/63/EU.
The rats will be euthanized at 4 or 8 weeks after start of the experiment with an overdose of CO_2 inhalation.

Rabbits:

Surgical procedure- The left hind leg is shaven and disinfected with povidone iodine. The stifle joint is opened with an incision through the skin and the patellar tendon. A hole is drilled into the tibia through the cartilage layer with a hand drill. The bacteria are inoculated into the cavity in a small volume with a pipette. A coated titanium implant is press-fit into the cavity, flushed with the cartilage surface. The patellar tendon and the skin are closed with resorbable sutures. The surgeries are estimated to take half an hour. In vivo micro-CT scanning is performed with appropriate anesthetics similar to the surgery (up to 3 times). Fluorochrome markers are injected subcutaneously to determine the onset and progression of bone formation (max. at 3 different time points). Blood is drawn from the ear vein (at max. 5 time points) to measure systemic markers of inflammation. The animals are euthanized after 4 and 8 weeks.

Anethesia- Combination of injection analgetics/anesthetics (in consultation with the designated veterinarian).

Pain management- Injection analgetics (usually buprenorphine with 12 hour interval), starting before surgery and continued after surgery (in consultation with the designated veterinarian). If any signs of pain continue, pain medication will be prolonged.

Antiseptic techniques- Surgery will be performed under aseptic conditions. The skin will be disinfected with povidone iodine. The surgeon will wear scrubs, sterile gloves, sterile gown, and a surgery mask/cap. Only autoclaved instruments will be used.

Postoperative care- The animals will be placed on heat blankets. The animals will be housed per two if possible. The animals will be scored daily for well-being in the first week and weekly thereafter. The rabbits will receive special food (soaked in water) when food intake is decreased (e.g. by systemic inflammation and as a side effect of the pain medication).

Infection measurements- In vivo scanning (under injection analgetics/anesthetics) is performed for a maximum of 3 times in total. Bacterial culture of bone and implant after euthanization is the primary outcome measure for bacterial killing. Systemic markers of inflammation are measured on blood samples.

Bone formation measurements- Bone-implant contact as determined on histology and aforementioned in vivo scans. The incorporation of fluorochrome markes will be determine by fluorescence microscopy to assess the onset and progression of new bone formation.

Euthanasia protocol- One of the methods listed in appendix IV of directive 2010/63/EU.
The rabbits will be euthanized after 4 and 8 weeks after the start of the experiment (usually with an overdose of Pentobarbital i.v.). This will be done under general anesthetics and pain medication, similar to the protocol used during surgery.

Geef aan welke overwegingen en statistische methoden worden gebruikt om het aantal benodigde dieren tot een minimum te beperken.

The following section describes the minimal number of animals needed for each study. For each study, a percentage of animals are added to the total to account for a possible loss of animals during the experiment. The percentage loss of animals is based on literature (rat experiments) or on our own experience with the same model and inoculation dose (rabbit experiments). Based on literature, ahigher percentage loss is expected in rats (10%) compared to rabbits (5%). Furthermore, we expect a higher percentage loss in the pilot study performed in rats, due to a higher chance of hematogenous infection. A pilot study has already been performed for the rabbit model by our group in the past.

STUDY 1, pilot study in rats (24 rats)
A pilot study will be performed to determine the minimal dose of S. aureus needed to get a chronic infection of the implant [1]. Although these doses are also described in literature [1], different experimental setting (bacterial culture conditions, materials, housing conditions) may affect the results. Our goal is not to demonstrate statistical significance differences. We believe the chosen group sizes will allow for the identification of the optimal bacterial dose. The minimal dose for which all three rats show the presence of bacteria in the bone after 4 weeks will subsequently be used to test the efficacy of the coatings.
Due to the relative higher chance of hematogenous infection for the higher CFU doses in this pilot study, a 20% of rats (4 rats) are added to the total to account for possible loss of animals.

1) Neg control (N=2)
2) 10^2 CFU S. aureus (N=3)
3) 10^3 CFU S. aureus (N=3)
4) 10^4 CFU S. aureus (N=3)
5) 10^5 CFU S. aureus (N=3)
6) 10^6 CFU S. aureus (N=3)
7) 10^7 CFU S. aureus (N=3)

For the following experiments, we will make an estimation of the the expected difference of the means and coefficient of variance will be based on the in vitro observations: 50% expected difference of the means and 50% coefficient of variance in bacterial killing. We will perform a one way ANOVA's with a Bonferroni post hoc correction. The alpha will be adjusted for the multiple comparions of the experimental groups (specified next per individual experiment). A two sided test will be used. We will use the S. aureus dose as determined in the pilot study.

STUDY 2, the effect of inorganic nanoparticles (73 rats):

1) Untreated implant (N=11)
2) Implant with nanotubes (NT) (N=11)
3) Implant with NT + Silver (AgNO3, 0.5 M) (N=11)
4) Implant with NT + Silver (AgNO3, 1.5 M) (N=11)
5) Implant with NT + Zinc (Zn(NO3)2, 0.015 M) (N= 11)
6) Implant with NT + Zinc (Zn(NO3)2, 0.030 M) (N= 11)

The best performing concentration of each element (3/4 and 5/6) is compared to the NT control (group 2).
Using an adjusted 'alpha' of 0.025 (0.05/2), a power of 80%, and a two-sided test, we find that 11 animals are needed per group when considering the difference of the means and a coefficient of variance of both 50%. We add an extra 7 animals to account for a possible loss of animals during the experiment. We believe that a loss of approximately 10%, due to systemic effects of infection, is a realistic estimation [2,3,4].

STUDY 3, the effect of controlled release of the inorganic nanoparticles (e.g. silver and zinc) (97 rats):

1) Implant with nanotubes (NT) (N=11 x 4 and 8 weeks)
2) Implant with NT + hydrogel (N=11 x 4 and 8 weeks)
3) Implant with NT + zinc in hydrogel (N=11 x 4 and 8 weeks)
4) Implant with NT + silver in hydrogel (N=11 x 4 and 8 weeks)

Groups 3 and 4 are compared to group 2 at the two different time points (no repeated measurements).
Using an adjusted 'alpha' of 0.025 (0.05/2), a power of 80%, and a two-sided test, we find that 11 animals are needed per group when considering the difference of the means and a coefficient of variance of both 50%. We add an extra 9 animals to account for a possible loss of animals during the experiment. We believe that a loss of approximately 10%, due to systemic effects of infection, is a realistic estimation [2,3,4].

STUDY 4, the effect of layer-by-layer release of antimicrobials (e.g. gentamicin) and growth factors (e.g. BMP-2) (100 rats).
1) Untreated implant (N=13)
2) Implant + growth factor (e.g. BMP-2) low concentration (N=13)
3) Implant + growth factor (e.g. BMP-2) high concentration (N=13)
4) Implant + antimicrobial (e.g. gentamicin) low concentration (N=13)
5) Implant + antimicrobial (e.g. gentamicin) high concentration (N=13)
6) Implant + growth factor (e.g. BMP-2)/antibiotic (e.g. gentamicin) low concentration (N=13)

7) Implant + growth factor (e.g. BMP-2) /antibiotic (e.g. gentamicin) high concentration (N=13)

The best layer-by-layer designs will be compared to the effect of the individual components (group 6 vs. group 2 and 4; group 7 vs. group 3 and 5). Thus, 4 comparisons are made. Using an adjusted 'alpha' of 0.0125 (0.05/4), a power of 80%, and a two-sided test, we find that 13 animals are needed per group when considering the difference of the means and a coefficient of variance of both 50%. We add an extra 9 animals to account for a possible loss of animals during the experiment. We believe that a loss of approximately 10%, due to systemic effects of infection, is a realistic estimation [2,3,4].

STUDY 5, the effect of the optimal bacterial-killing and bone promoting coatings (176 rabbits)

1) Untreated implant (N=12 x two time points)
2) Implant with nanotubes (N=12 x two time points)
3) Implant with Zinc or Silver (N=12 x two time points)
4) Implant with Zinc or Silver in hydrogel (N=12 x two time points)
5) Implant with growth factor (e.g. bone morphogenetic protein 2) (N=12 x two time points)
6) Implant with antimicrobial (e.g. gentamicin) (N=12 x two time points)
7) Implant with growth factor (e.g. bone morphogenetic protein 2)/antimicrobial (e.g. gentamicin) in layer-by-layer design (N=12 x two time points).

At each time point, group 4 is compared to group 3, and group 7 is compared to group 5 and 6 (no repeated measures). Thus, 3 comparisons are made. Using an adjusted 'alpha' of 0.0167 (0.05/3), a power of 80%, and a two-sided test, we find that 12 animals are needed per group when considering the difference of the means and a coefficient of variance of both 50%. We add an extra 8 animals to account for a possible loss of animals during the experiment. Based on our previous experience with this model (DEC 2013.III.11.083), we believe that a loss of approximately 5% of the rabbits, due to the systemic effects of infection, is a realistic estimation.

In total, we will therefore need 294 rats and 176 rabbits.

REFERENCES

[1] Reizner, JG Hunter, NT O'Malley, RD Southgate, EM Schwarz, SL Kates. A systematic review of animal models for Staphylococcus aureus osteomyelitis. Eur Cell Mater. 2014 Mar 25;27:196-212.
[2] Lucke et al. A new model of implant-related osteomyelitis in rats. J Biomed Mater Res B Appl Biomater. 2003 Oct 15;67(1):593-602.
[3] Poeppl et al. Daptomycin, Fosfomycin, or Both for Treatment of Methicillin-Resistant Staphylococcus aureus Osteomyelitis in an Experimental Rat Model. Antimicrob Agents Chemother. 2011 Nov; 55(11): 4999–5003.
[4] Poeppl et al. Efficacy of Fosfomycin Compared to Vancomycin in Treatment of Implant-Associated Chronic Methicillin-Resistant Staphylococcus aureus Osteomyelitis in Rats. Antimicrob Agents Chemother. 2014 Sep; 58(9): 5111–5116.

B. De dieren

Benoem de diersoorten, herkomst, geschatte aantallen en levenstadia. Onderbouw deze keuzes.

Rats:
Male Sprague Dawley, age 12-16 weeks (Charles River) . We expect to use 279 rats (see Statistical Methods).

The rat is a good animal model for our screening studies due to their small size, low cost, and established models of developing osteomyelitis described in literature [1,2]. Sprague Dawley rats have been used most often. By selecting the Sprague Dawley rats, we can base our bacteria inoculation dose on the literature.
Male rats are preferred of their larger size, which allows for larger K-wires to be implanted into the tibia, offering a larger surface area of the coating. The age of the rats is subsequently selected to ensure enough space in the tibial cavity for the placement of the k-wires (0.8 Ø x 25). Furthermore, the estrogen levels in female rats can fluctuate due to stress and age [3], and can affect bone regeneration [3,4]. Futhermore, it has been shown that male an female rats show a difference in their bone metabolism [5, 6,7]. If male and female rats are studied in a mixed population, this will cause a strong increase in the observed variance. We therefore prefer to use male rats only. The male rats can be housed together without problems.

Rabbits:
Female New Zealand White, age 12-16 weeks (Charles River). We expect to use 176 rabbits (see Statistical Methods).

Our group has previously performed dose-response studies with S. aureus in this adolescent rabbit model. Female rabbits can be used, which allows for grouped housing. Although, based on literature, both male and female rabbits can be used for this kind of research, we would like to use a single sex of rabbits. First, this minimizes the variance in the experiments, allowing the use of less animals. Second, we can compare our data to the results we obtained previously with this model. The selected age of the rabbits allows for the implantation of more clinically relevant-size implants (4 mm Ø x 25 mm). Furthermore, the adolescent age of the rabbits allows for in vivo scanning as this is problematic for larger animals, To our best knowledge, current literature does not suggest that adolescent rabbits may be more suitable as an osteomyelitis model than mature rabbits, or vice versa.

REFERENCES

1. W. Reizner, J.G. Hunter, N.T. O'Malley, R.D. Southgate, E.M. Schwarz, and S.L. Kates. A systematic review of animal models for Staphylococcus aureus osteomyelitis. Eur Cell Mater. 2014; 27: 196–212.
2. Patel M, Yuri Rojavin, Amir A. Jamali, Samantha J. Wasielewski, and Christopher J. Salgado. Animal Models for the Study of Osteomyelitis. Semin Plast Surg. 2009 May; 23(2): 148–154.
3. Hong L, Sultana H, Paulius K, Zhang G. Steroid regulation of proliferation and Biochem Mol Biol. 2009 Apr;114(3-5):180-5. PubMed PMID: 19429449. Pubmed Central PMCID: 2682591.
4. Calis M, Demirtas TT, Atilla P, Tatar I, Ersoy O, Irmak G, et al. Estrogen as a novel agent for induction of adipose-derived mesenchymal stem cells for osteogenic differentiation: in vivo bone tissue-engineering study. Plastic and reconstructive surgery. 2014 Apr;133(4):499e-510e. PubMed PMID: 24675202.
5. Sample SJ, Racette MA, Hao Z, Thomas CF, Behan M, Muir P. Functional adaptation in female rats: the role of estrogen signaling. PloS one. 2012;7(9):e43215. PubMed PMID: 22984413. Pubmed Central PMCID: 3439425.
6. Strube P, Mehta M, Baerenwaldt A, Trippens J, Wilson CJ, Ode A, Perka C, Duda GN, Kasper G. Sex-specific compromised bone healing in female rats might be associated with a decrease in mesenchymal stem cell quantity. Bone. 2009 DecL45(6):1065-72

C. Hergebruik

Is er hergebruik van dieren?

☒ Nee, ga door met vraag D.

☐ Ja > Geef aan op basis van welke overwegingen hergebruik in dit geval acceptabel wordt geacht.

Is er in het voorgaande of in het geplande gebruik sprake van (of een risico van) ernstig ongerief?

☒ Nee

☐ Ja > Geef aan op basis van welke overwegingen hergebruik in dit geval acceptabel wordt geacht.

D. Vervanging, vermindering en verfijning

Laat zien hoe de toepassing van methoden voor vervanging, vermindering en verfijning zijn meegewogen bij het bepalen van de experimentele strategie, de keuze van de dieren en de opzet van de dierproef en welk keuzes daarbij zijn gemaakt.

Replacement:
The effects of the implant coatings are first studied in vitro. For this purpose, we use a cytotoxicity assay. Also, bacterial proliferation and biofilm formation can be assessed in vitro. The best-performing conditions will subsequently be tested in vivo, as indirect effects by immune cells is hard to mimick in an in vitro model.

Reduction:
For each experiment, a power analysis will be performed to determine the minumum number of animals required. The inter-animal variation will be minimized so that smaller group sizes are needed to reach statistical significance. Rats with the same sex and age will be used.
By performing in vivo scanning in the animals, the effects of the infection on bone changes can be assessed at different time points without the need of different animals for different time points (not possible if bacterial culture has to be performed at different time points).

Refinement:
- Animals will be given 1 week to acclimatize to their new environment
- Animals have cage enrichment
- Animals are weighed weekly to monitor welfare
- Animals receive appropriate analgetics and anaesthetics (in consultation with the designated veterinarian)
- The animals are housed together as long as no signs of agression towards each other exist
- Daily scoring of the animals will be performed after the surgeries. Pain medication will be continued if necessary. For rabbits, this is a standard procedure. For rats, the extra handling needed for scoring will be requested in the working protocol
- In vivo scanning will be performed under adequate anaesthetics (in consultation with the designated veterinarian)

- During and after surgery, the animals will be placed on heat blankets
- Eye ointment will be used to prevent dry eyes of the animals during surgery
- Skin wounds will be sutured intracutaneously to prevent opening

Geef aan welke maatregelen zijn genomen om de kans op pijn, lijden of angst bij de dieren en de kans op nadelige milieueffecten tot een minimum te beperken.

The animals will have at least 1 week to acclimatize to the new environment in the GDL. The animals will be housed per two as much as possible. The respiration of the rats will be monitored by the researcher during surgery. The rabbits will be monitored for respiratory and heart function by the designated biotechnician. The animals will be placed on heat blankets after surgery and returned to routine housing after recovery of anethesia. Unrestricted weight bearing and activity will be allowed after surgery. Food is given ad libitum. The animals will be scored weekly by the animal caretakers or the researcher (in consultation with each other). After surgeries, this frequency will be increased. In the case of unexpected complications, the animal caretakers and the designated veterinarian are consulted.

Herhaling en duplicering

E. Herhaling

Geef aan hoe is nagegaan of deze dierproeven niet al eerder zijn uitgevoerd. Indien van toepassing geef aan waarom duplicatie noodzakelijk is.

These coating methods have been newly developed by our group. With the current literature, we are unable to predict the performance of our coating methods in an in vivo model.

Huisvesting en verzorging

F. Huisvesting en verzorging

Worden de dieren anders dan volgens de eisen in bijlage III van de richtlijn 2010/63/EU gehuisvest en/of verzorgd?

☒ Nee

☐ Ja > Geef, indien dit kan resulteren in nadelige effecten op het dierenwelzijn, aan op welke wijze de dieren worden gehuisvest en verzorgd en motiveer de keuze om af te wijken van de eisen in bovengenoemde bijlage III.

G. Plaats waar de dieren worden gehuisvest

Worden de dierproeven geheel of gedeeltelijk uitgevoerd bij een inrichting die niet onder de rechtstreekse verantwoordelijkheid van een instellingsvergunninghouder Wod valt?

☒ Nee > Ga verder met vraag H.

☐ Ja > Geef aan wat voor bedrijf of instelling dit betreft.

Waarom is hiervoor gekozen en hoe wordt een adequate huisvesting, verzorging en behandeling van de dieren gewaarborgd?

Ongeriefinschatting / humane eindpunten

H. Pijn en pijnbestrijding

Valt te voorzien dat er pijn kan optreden bij de dieren?

☐ Nee > Ga verder met vraag I.

☒ Ja > Worden in dat geval verdoving, pijnstilling en/of andere pijnverlichtingsmethoden toegepast?

☐ Nee > Motiveer dan waarom geen pijnverlichtingsmethoden worden toegepast.

☒ Ja

I. Overige aantasting van het welzijn en maatregelen

Welke eventuele andere vormen van welzijnsaantasting worden voorzien?

Moderate stress (cumulative) is estimated based on the following procedures:
- Moderate stress due to the surgery. Animals will experience pain in the treated joint. The animals might avoid loading of the limb after surgery. Bacterial infection casuses local and systemic signs of inflammation. Animals will have reduced food intake and experience weight loss after surgery. In previous experiments, the animals were loading the affected limb a few days after surgery.
- Analgetics: mild stress. Animals experience lameness and reduced food intake due to the injection analgetics.
- Scoring of the animals after surgery: mild stress. Extra handling of the animals for weighing and scoring
- In vivo scanning (max. 3 times depending on the duration of the experiment): mild to moderate stress. Due to handling and anesthetics.
- Euthanasia: mild stress due to handling
- Blood harvesting (rabbits, max. 5 times depending on the duration of the experiment): moderate stress due to local ear irritation and stress during handling (procedure is mild, but is considered as moderate because it is repeated)
- Fluorochrome injections (max. 3 times depending on the duration of the experiment): moderate stress due to local skin irritation and stress during han dling (procedure is mild, but is considered as moderate because it is repeated)

Geef aan wat de mogelijke oorzaken hiervan zijn.

All described measurements are needed to create the least harm for the animals and most secure outcomes for this project.

Beschrijf welke maatregelen worden genomen om deze schadelijke effecten te voorkomen of waar mogelijk te minimaliseren.

1. At least 1 week to acclimatize to the new environment
2. Adequate use of analgetics (in consultation with the designated veterinarian)
3. Adequate use of anesthetics (in consultation with the designated veterinarian)
4. Observation of vital signs during surgery.

rats: depth or narcosis, respiration, tail pinch test
rabbits: monitoring of respiratory and heart function by the biotechnical. The depth or narcosis (twitching/movement) can additionally be observed by the researcher performing the surgery.

5. Scoring of the animals daily after surgery
6. Euthanasia according to one of the methods listed in Appendix IV of directive 2010/63/EU.
7. Pain medication, starting before surgery, and continued for two days standard. If there is any indication, pain medication will be continued beyond this period.

J. Humane eindpunten

Valt te voorzien dat zich bij deze dierproef omstandigheden voordoen waarbij het toepassen van humane eindpunten geïndiceerd is om verder lijden van de dieren te voorkomen?

☐ Nee > Ga verder met vraag K.

☒ Ja > Geef aan welke criteria hierbij worden gehanteerd.

- The rats will be euthanized in case one or more of the following humane endpoints are observed:
  - Weight loss (criteria determined in consultation with the veterinarion)
  - Severe lethargy
  - Severe tachycardia
  - Severe tremor
- Possible other signs of sepsis
- Loss of the animals' ability to walk and feed themselves
- Unexpected wound complications (such as non-closing and severe pus formation)

- The rabbits will be euthanized in case one or more of the following humane endpoints are observed:
  -Weight loss (criteria determined in consultation with the designated veterinarian)
  - Severe lethargy
  - Severe tachycardia
  -Severe tremor
  - Possible other signs of sepsis
  - Loss of the animals' ability fo walk and feed themselves
  -Joint infection with severe pus formation.

Welk percentage van de dieren loopt kans deze criteria te halen?

Rats: unlikely, max. 10%. The chance is higher in the pilot study, max. 20%.
Rabbits: very unlikely, max. 5%.

K. Classificatie van ongerief

Geef aan hoe in het licht van alle hierboven beschreven negatieve effecten het cumulatief ongerief wordt geclassificeerd in termen van '*terminaal*', *'licht'*, '*matig'* of *'ernstig'* ongerief.

The procedure is classified as moderate.

Einde experiment

L. Wijze van doden

Worden de dieren als onderdeel van het experiment of na afloop van het experiment gedood?

☐ Nee > Ga verder met de ondertekening.

☒ Ja > Geef aan waarom het doden van dieren als eindpunt essentieel is voor deze proef.

The animals need to be euthanized to harvest the bone and implant for bacterial culture.

Wordt er een methode(n) van doden uit bijlage IV van richtlijn 2010/63/EU toegepast?

☐ Nee > Beschrijf de euthanasiemethode en onderbouw de keuze hiervoor.

☒ Ja

Bijlage
Beschrijving dierproeven

- Deze bijlage voegt u bij uw projectvoorstel dierproeven.
- Per type dierproef moet u deze bijlage invullen en toevoegen.
- Meer informatie vindt u op de website www.zbo-ccd.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Vul uw deelnemernummer van de NVWA in.	11500
1.2	Vul de naam van de instelling of organisatie in.	UMC Utrecht
1.3	Vul het volgnummer en het type dierproef in. *Gebruik de volgnummers van vraag 3.4.4 van het format Projectvoorstel.*	Volgnummer: 2 Type dierproef: Spinal fusion in rats and rabbits in combination with ectopic implants

2 Beschrijving dierproeven

A. Experimentele aanpak en primaire uitkomstparameters

Beschrijf de keuze van de experimentele aanpak en de primaire uitkomstparameters.

We have previously identified inflammation-associated cytokines and bacterial-derived factors that could serve as bone-stimulating coatings on ceramics or metals intended for various orthopedic applications [1,2,3]. We more recently also showed that the use of components derived from bacteria is potentially a safe and effective way to stimulate bone formation (unpublished). These biological factors enhance bone formation by causing a beneficial immune response.

Currently, the transplantation of patient's own bone is used to stimulate bone formation in patient's when needed, including non-healing fractures, filling of large bone defects, or the re-alignment of the skeleton [4]. There are however a lot of complications associated with the bone harvesting procedure and there

is only a limited amount of bone available for the surgeon. The coating of materials with the aforementioned biological factors could be an alternative way to stimulate de novo bone formation or to improve the bone integration of implants. The fusion of vertebrae- e.g. during abnormal curvature of the spine or due to chronic back pain- is the most important application for which bone grafts are needed [6].

We aim to test the efficacy of our coatings on the bone formation in a spinal fusion model in rats and rabbits [7, 8]. As spinal fusion is the most important application for which bone grafts are used, we would like to test the efficacy of our coatings in the same setting. A calcium phosphate ceramic will be used (e.g. biphasic calcium phosphate BCP or tricalcium phosphate TCP). These materials are promosing for use in spinal fusion because of their ability to conduct bone formation along their surface. If bone-stimulating coatings are identified in the spinal fusion model, these can directly be tested for their ability to improve bone integration in the implant infection models.

The spinal fusion procedure is relatively simple to perform in these animals, in contrast to the spinal fusion technique in humans or larger animal models, where instrumentation is required. The rat spinal fusion model allows for better study of the underlying processes as there are more research tools available for rodents compared to rabbits and larger animals. The rabbit model on the other hand allows for better translation to the clinic, as larger constructs can be tested and the immune system is more comparable to humans. When using inflammation-associated cytokines or components derived from bacteria, the immune response is an important outcome parameter. We believe that this is more representative of the human situation in rabbits than it is in rats. Furthermore, in rabbits, a comparison can be made between our coating method and the current gold standard in the clinic (e.g. autologous bone harvested from the hip bone). This is difficult to perform in rats due to their smaller size.

The implantation of ectopic constructs in the same animals allows for additional studies to be performed in parallel to the functional spinal fusion test in the orthotopic location.
First, in an ectopic location, bone formation is only the result of osteoinduction (i.e. differentiation of stem cells into bone cells), whereas osteoconduction (i.e. bone outgrowth from existing bone) also plays a role in the orthotopic location. By combining the ectopic and orthotopic sites, the potential of the inflammatory compounds for osteoinduction and osteocondution can be both studied. This can elucidate the potential use of the compounds in different applications and locations.
Second, the optimal dose of in vitro-indentified inflammatory compounds can be studied in an ectopic location before testing them in an orthotopic location. Because there are more implantations possible in the ectopic site compared to the orthotopic site, we can also try to identify new bone-stimulating inflammatory compounds in the ectopic location which can be tested in follow-up studies. For the first in vivo studies, the optimal dose of interesting inflammatory compounds have already been determined ectopically in a previous study and can be directly tested in an orthotopic location.
By combining the ectopic and orthotopic locations, the animals are used in an optimal way and multiple research questions can be answered within the same study.

Several outcome parameters will be studied related to new bone formation and inflammation. In vivo micro-CT scans are performed under general anesthesia to quantify new bone formation at different time points. Fluorochrome markers are injected at different time points to trace the onset and progression of bone formation. After euthanization of the animals, local inflammation markers are measured on tissue samples. For the ectopic implants, quantification of the bone staining in histological samples is performed. For the spinal fusion implants, a combination of different methods is used to assess the degree of fusion between the vertebrae, including manual palpation, ex vivo micro-CT imaging, and quantification of the bone tissue in histological samples. In the rabbit studies, the systemic effects of the coatings will also be assessed by measuring systemic markers of inflammation on blood samples. Local inflammation markers are also measured on the spinal fusion masses.

References:

1. M Croes, F. Cumhur Oner, Moyo C. Kruyt, Taco J. Blokhuis, Okan Bastian, Wouter J.A. Dhert, Jacqueline Alblas. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. Plos one 2015.
2. M Croes, W Boot, B Pouran, MC Kruyt, D Gawlitta, Y van der Helm, C Vogely, H Weinans, J Alblas, WJA Dhert, FC Oner. Bacterial infection increases bone volume in a rabbit osteomyelitis model. 2015. Submitted
3. M Croes, W Boot, MC Kruyt, WJA Dhert, B Pouran, FC Oner, J Alblas. Bacteria-associated inflammation induces bone formation in a pre-clinical rabbit model. In preparation
4. PV Giannoudis, H Dinopoulos, E Tsiridis : Bone substitutes: An update. Injury 2005.
5. BM Desai. Osteobiologics. Am J Orthop 2007
6. WG De Long, TA Einhorn, K Koval et al. Current concepts review: Bone grafts and bone graft substitutes in orthopaedic trauma surgery. J Bone Joint Surg AM 2007
7. SD Boden, GJ Martin, M Morone, JL Ugbo, L Titus, WC Hutton. The use of coralline hydroxyapatite with bone marrow, autogenous bone graft, or osteoinductive bone protein extract for posterolateral lumbar spine fusion. Spine. 1999;24:320–327
8. Drespe et al. Animal models for spinal fusion. Spine J. 2005.

Beschrijf de beoogde behandeling van de dieren (inclusief de aard, de frequentie en de duur van de behandelingen waaraan de dieren worden blootgesteld) en onderbouw de gekozen aanpak.

Surgical procedures

Ectopic implantations: Using a single skin midline incision in the dorsum, PMMA (poly methyl methacrylate) discs are implanted intramuscularly and subcutaneously. We have previously used up to 18 samples in rabbits and up to 14 samples in rats, leaving enough space beteen the samples to minimize cross-over effects between samples. After 6 weeks, all PMMA discs are removed from their surrounding membranes ('biomembranes'), and replaced scaffolds (calcium phosphate ceramics or porous metal implant) coated with the factors of interest. These 'biomembranes' act as a physical barrier, preventing initial leakage of growth factors or inflammation-associated factors to the surrounding tissues [1]. As a results, one creates a more localized inflammatory response and limits the cross-over effects to neighbouring implants. The first procedure (implantation of the spacer) is expected to take approximately 1 hour, while the second procedure (implantation of the loaded material in the 'biomembrane') is thought to take approximately 1.5 hours. The implantation of the loaded constructs is always performed in a randomized way.

The membranes act as a physical barrier, preventing initial leakage of the delivered components which could effect neighbouring scaffolds. The skin wounds are closed with resorbable sutures. During the experiment, fluorochrome markers are injected subcutaneously at max. three time points to determine the progression of bone formation by fluorescence microscopy. In vivo micro-CT scans are performed under general anesthesia to quantify the progress of bone formation (max. 3 times). The animals are euthanized 8-12 weeks after the second surgery to retrieve the implants for analyses by histology. This exact end point (8-12 weeks) is chosen for each experiment separately, but is determined by the speed of bone formation which is expected (i.e. if bone stimulating growth factors such as BMPs are used), and will be minimized.

This model has been used by our group in both species successfully without loss of animals. In rabbits, blood is harvested from the ear vein at max. 5 time points to measure systemic markers of inflammation.

Spinal fusion: this procedure is combined with the second surgery for the ectopic implantations at week 6. The dorsal midline skin incision is followed by two paramedian fascial incisions between the multifidus and longissimus muscles until the transverse processes in the lumbar region are exposed. The exposed transverse processes are decorticated bilaterally with an electric burr. On both sides of the vertebra, the graft of interest is implanted next to the spine. This involves a calcium phosphate ceramic or a porous metal implant in the experimental group, with or without coating with the biological factor of interest. When a comparison is made with the current gold standard in the clinic (i.e. autologous bone), separate fascial incisions are used to harvest corticocancellous bone from the iliac crest bone to place between the transverse processes in the same way. The spinal fusion procedure can be performed at a single level or at two levels of the vertebrae [1,2, 3]. The skin wound is closed with resorbable sutures. The animals are euthanized (see ectopic implantations) for ex vivo micro-CT scanning and the fusion masses/ectopic implants are retrieved for histological analyses. Local inflammation markers are measured in the fusion masses.

Bone regeneration measurements

The primary outcome parameter is the amount of bone formation after 8-12 weeks. For the ectopic implants, quantification of the bone in histological samples is the best method. For the spinal implants, a combination of techniques is used to assess the degree of fusion between the vertebrae, including manual palpation, in vivo and ex vivo micro-CT, and quantification of the bone tissue in histological samples. Animals will receive fluorochrome markers subcutaneously to determine the onset and localization of new bone formation by fluorescence microscopy post-mortem.

Inflammation measurements

In the rabbits, blood will be harvested at max. 5 time points to measure systemic markers of inflammation. Local inflammation markers will be measured on tissue samples after euthanization.

Animal stress

The animals are estimated to experience mild stress (cumulative). The procedures are expected to take approximately 1 hour (first surgery) and 2 hours (second surgery).

Anaesthesia protocol

Rats: Start and maintained with isoflurane/oxygen.
Rabbits: a combination of injection analgetics/anesthetics is used. The protocols for adequete anaesthesia are developed together with the veterinarian.

Pain management

All animal receive injection analgetics. The protocols used for pain management will be developed together with the designated veterinarian. If any

unexpected signs on pain continue to exist in the first days after the operational procedure, as observed during daily scoring of the animals, the designated veterinarian will be consulated for the adequate pain management.

Antiseptic techniques

All operations will be performed aseptically conditions. The surgeon will wear scrubs, sterile gloves and sterile gown. The surgeon will wear a surgery mask and cap. Only autoclaved instruments will be used. After shaving, the skin will be disinfected with Providone Iodine. All materials introduced into the animals are autoclaved or filtered to ensure sterility.

Postoperative care

The animals will be placed on heat blankets post-operatively. The animals will be housed per two, unless there are signs of agression towards each other. The animals will be scored daily for one week after the operational procedure. The weight of the animals will be measured once a week. The animals will receive injection pain medication for two days at minimum, and longer if necessary.

Euthanasia protocols

Euthanasia is performed according to one of the methods listed in appendix IV of directive 2010/63/EU. The animals will be euthanized 8-12 weeks after the second surgery . This involves CO2 inhalation in rats, and an overdose of Pentobarbital i.v. in rabbits under the same general anaesthesia and pain medication as the surgery procedure.

References:

1. Viateau V, Guillemin G, Bousson V, Oudina K, Hannouche D, Sedel L, et al. Long-bone critical-size defects treated with tissue-engineered grafts: a study on sheep. J Orthop Res 2007;25: 741-9
2. SD Boden, GJ Martin, M Morone, JL Ugbo, L Titus, WC Hutton. The use of coralline hydroxyapatite with bone marrow, autogenous bone graft, or osteoinductive bone protein extract for posterolateral lumbar spine fusion. Spine. 1999;24:320–327
3. AM Riordan, R Rangarajan, JW Balts, WK Hsu, PA Anderson. Reliability of the Rabbit Postero-Lateral Spinal Fusion Model: A Meta-Analysis. JOR 2013
4. Drespe et al. Animal models for spinal fusion. Spine J. 2005.

Geef aan welke overwegingen en statistische methoden worden gebruikt om het aantal benodigde dieren tot een minimum te beperken.

The following section describes the minimal number of animals needed for each study. For the rabbit study, a 5% loss of animals is estimated. This percentage is based on literature. As this loss in the rabbits is mainly the result of the autologous bone harvesting procedure, it is not expected in the rat studies.

The outcome parameter on which the group size are based is the success of spinal fusion (yes or no) as determined by micro-CT. The difference of the means and the coefficient of variance of treatment with a biological coating in the spinal fusion model is unknown [1]. We can therefore only determine the minimal

group size based on a rough estimation of these parameters.

In a typical experimental design, 3 groups are used. We will perform a one way ANOVA's with a Bonferroni post-hoc correction. The alpha is adjusted according to two comparisons that are made (experimental group vs. positive control and negative control OR two experimental groups vs. positive control). For example:

1) Positive control group, autograft or bone morphogenetic protein (BMP)
2) Negative control group (e.g.calcium phosphate ceramic such as biphasic calcium phosphate BCP or tricalcium phosphate TCP)
3) Experimental group (e.g. calcium phosphate ceramic coated with proinflammatory cytokine or bacterial-derived factor)

Using an adjusted 'alpha' of 0.025 (0.05/2), a power of 80%, and a two-sided test, we find that 11 animals are needed per group when considering the difference of the means and a coefficient of variance of both 20%. We hereby consider a difference of the means of at least 20% to be of clinically relevance.

We expect to perfom the following studies in the upcoming 5 years:

STUDY 1, the effect of inflammation-associated cytokines on bone formation in rats (33 rats):
Group 1. Pos control, calcium phosphate with BMP, N=11
Group 2. Neg control, empty calcium phosphate (e.g. BCP or TCP), N=11
Group 3. Experimental group, calcium phosphate coated with proinflammatory cytokines, N=11

We have already determined the optimal concentration of a number inflammatory compounds in an ectopic location. These can be studied in the orthotopic location in study 1. Simultaneously, we can perform dose studies for bacterial components determined in vitro in the ectopic location in the same animals. These can then be tested in the orthotopic location in study 2.

STUDY 2, the effect of bacterial components on bone formation in rats (33 rats):
Group 1. pos. control, calcium phosphate with BMP, N=11
Group 2. Experimental group 1, calcium phosphate coated with bacterial component 1, N=11
Group 3. Experimental group 2, calcium phosphate coated with bacterial component 2, N=11

The optimal concentration of bacterial components are determine in the ectopic location in study 1. These can be studied in the orthotopic location in study 2. Simultaneously, we can perform dose studies for future experiments in the ectopic location in study 2.

STUDY 3, the effect of inflammation-associated cytokines on bone formation and systemic inflammation markers in rabbits (35 rabbits):
Group 1. Pos control, autologous bone, N=11
Group 2. Neg control, empty calcium phosphate, N=11
Group 3. Experimental group, calcium phosphate coated with inflammation-associated cytokines, N=11

For this rabbit study, we add an extra 2 animals to account for a possible loss of animals during the experiment. We believe that a loss of approximately 5%,

due to deep wound infections or autologous bone harvest-associated nerve damage and blood loss, is a realistic estimation [2-6].

We have already determined the optimal concentration of a number inflammatory compounds in an ectopic location. These can be studied in the orthotopic location in study 3. Simultaneously, we can perform dose studies for bacterial components determined in vitro in the ectopic location in the same animals. Subsequently, the optimal dose can be tested in the orthotopic location in study 4.

STUDY 4, the effect of bacterial components on bone formation and systemic inflammation markers in rabbits (35 rabbits) :

Group 1. Pos control, autologous bone, N=11
Group 2. Experimental group 1, calcium phosphate coated with bacterial component 1, N=11
Group 3. Experimental group 2, calcium phosphate coated with bacterial component 2, N=11

For this rabbit study, we add an extra 2 animals to account for a possible loss of animals during the experiment. We believe that a loss of approximately 5%, due to deep wound infections or autologous bone harvest-associated nerve damage and blood loss, is a realistic estimation [2-6].

The optimal concentration of bacterial components are determine in the ectopic location in study 3. These can be studied in the orthotopic location in study 4. Simultaneously, we can perform dose studies for future experiments in the ectopic location in study 4.

Thus, we estimate that in total 66 rats and 70 rabbits are needed.

REFERENCES

1. AM Riordan, R Rangarajan, JW Balts, WK Hsu, PA Anderson. Reliability of the Rabbit Postero-Lateral Spinal Fusion Model: A Meta-Analysis. JOR 2013
2. Bransford et al. Effect of zoledronic acid in an L6–L7 rabbit spine fusion model. Eur Spine J. 2007 Apr; 16(4): 557–562.
3. Cinotti et al. Experimental posterolateral spinal fusion with porous ceramics and mesenchymal stem cells. J Bone Joint Surg Br. 2004 Jan;86(1):135-42.
4. Lee et al. The effect of acute smoking on spinal fusion: an experimental study among rabbits. J Trauma. 2005 Aug;59(2):402-8.
5. Lehman et al. The effect of alendronate sodium on spinal fusion: a rabbit model. Spine J. 2004 Jan-Feb;4(1):36-43
6. Long et al. The effect of cyclooxygenase-2 inhibitors on spinal fusion. J Bone Joint Surg Am. 2002 Oct;84-A(10):1763-8.

B. De dieren

Benoem de diersoorten, herkomst, geschatte aantallen en levenstadia. Onderbouw deze keuzes.

We would like to use both rats and rabbits for our experiments. There are more research tools (e.g. antibodies) available for the rat species, allowing for more mechanistic studies. The rabbits on the other hand allow for more translational research: safety studies can be performed due to their comparable immune system to humans, a comparison can be made between our coating and the golden standard (i.e. autologous bone grafting) and larger constructs can be tested in rabbits

Rats: male Fischer 344, 10-16 weeks (Charles River). We estimate to use 70 rats (see Statistical Methods).

The Fischer 344 rat has been used as a spinal fusion model before [1]. Our ectopic rat model also makes use of this male rat strain, wich allows for comparison of the data when using calcium phosphates or BMP-2. Furthermore, in this model we can make a good prediction of the amount of bone formation in the control group, which allows us to select the appropriate timepoints for analyses. The estrogen levels in female rats can fluctuate due to stress and age [2], and can affect bone regeneration [3,4]. Futhermore, it has been shown that male an female rats show a difference in their bone metabolism [5,6]. If male and female rats are studied in a mixed population, this will cause a strong increase in the observed variance. We therefore prefer to use male rats only. As an advantage, we have experienced that the male Fischer 344 rats can be housed together without problems [5,6]

Rabbits: adult female New Zealand White, 20-24 weeks (Charles River). We estimate to use 70 rabbits (see Statistical Methods).
The rabbit spinal fusion model has been used frequently, as the fusion rate with autologous bone is similar to humans. A number of characteristics of the rabbits (age, weight, gender, species) have been tested on the rate of fusion in a meta-analysis study [7]. This study shows that the use of female New Zealand white rabbits is very suitable. For our research this is beneficial, as we previously studied the biological coating components in female New Zealand white rabbits too. However, we wish to keep the sex within our spinal fusion model fixed, due to the 20% difference in the fusion rate between male and female rabbits [7]. The increase in variance when using a mixed-gender population would results in a need for a larger sample size to demonstrate a statistically significant difference. Individual housing of male rabbits is associated with a decreased general well-being, therefore the use of females may be preferable. There is no evidence that fluctuating estrogen levels in female rabbits can affect the bone formation process [8]. In addition, the rabbit tibia model also makes use of female rabbits, therefore allowing better translation to the spinal fusion model. This meta-analysis furthermore shows that the animals should be at least 3 kg in weight, while there seems to be less of an effect for the age of the animals. Our choice of the age/weight allows for a sufficient number of ectopic implants in the dorsal region, with enough space between them to minimal cross-over effects between the samples.

Adult rabbits are used as this seems to be beneficial for the success of fusion. Furthermore, almost all studies described in literature make use of adult rabbits [7].

Generally speaking, the rabbit model is used in a final step before an intrumented spinal fusion model (e.g. sheep or goats). As such, larger constructs can be used and the rabbit immune system allows for prediction of dosis effects considering that its immune system is more comparable to humans than rodents [9, 10]. This is an important aspect to consider, as some of the biological coatings induce bone formation through an immune response.

References:

1. Lopez et al. Acceleration of Spinal Fusion Using Syngeneic and Allogeneic Adult Adipose Derived Stem Cells in a Rat Model. J Orthop Res 2009.
2. Arakawa K, Arakawa H, Hueston CM, Deak T. Effects of the estrous cycle and ovarian hormones on central expression of interleukin-1 evoked by stress in female rats. Neuroendocrinology. 2014;100(2-3):162-77. PubMed PMID: 25300872.
3. Hong L, Sultana H, Paulius K, Zhang G. Steroid regulation of proliferation and Biochem Mol Biol. 2009 Apr;114(3-5):180-5. PubMed PMID: 19429449. Pubmed Central PMCID: 2682591.
4. Calis M, Demirtas TT, Atilla P, Tatar I, Ersoy O, Irmak G, et al. Estrogen as a novel agent for induction of adipose-derived mesenchymal stem cells for osteogenic differentiation: in vivo bone tissue-engineering study. Plastic and reconstructive surgery. 2014 Apr;133(4):499e-510e. PubMed PMID: 24675202.
5. Sample SJ, Racette MA, Hao Z, Thomas CF, Behan M, Muir P. Functional adaptation in female rats: the role of estrogen signaling. PloS one. 2012;7(9):e43215. PubMed PMID: 22984413. Pubmed Central PMCID: 3439425.
6. Strube P, Mehta M, Baerenwaldt A, Trippens J, Wilson CJ, Ode A, Perka C, Duda GN, Kasper G. Sex-specific compromised bone healing in female rats might

be associated with a decrease in mesenchymal stem cell quantity. Bone. 2009 DecL45(6):1065-72
7. AM Riordan, R Rangarajan, JW Balts, WK Hsu, PA Anderson. Reliability of the Rabbit Postero-Lateral Spinal Fusion Model: A Meta-Analysis. JOR 2013
8. Mapara M, Thomas BS, Bhat KM/ Rabbit as an animal model for experimental research. Dent Res J (Isfahan). 2012 Jan-Mar; 9(1): 111–118
9.Copeland S, Warren HS, Lowry, SF, Calvano SE, Remick D. Acute Inflammatory Response to Endotoxin in Mice and Humans. Clin Diagn Lab Immunol. 2005 Jan; 12(1): 60–67
10. Jacquier V, Estellé, Schmaltz-Panneau B et al. Genome-wide immunity studies in the rabbit: transcriptome variations in peripheral blood mononuclear cells after in vitro stimulation by LPS or PMA-Ionomycin. BMC Genomics 2015, 16:26

C. Hergebruik

Is er hergebruik van dieren?

☒ Nee, ga door met vraag D.

☐ Ja > Geef aan op basis van welke overwegingen hergebruik in dit geval acceptabel wordt geacht.

Is er in het voorgaande of in het geplande gebruik sprake van (of een risico van) ernstig ongerief?

☒ Nee

☐ Ja > Geef aan op basis van welke overwegingen hergebruik in dit geval acceptabel wordt geacht.

D. Vervanging, vermindering en verfijning

Laat zien hoe de toepassing van methoden voor vervanging, vermindering en verfijning zijn meegewogen bij het bepalen van de experimentele strategie, de keuze van de dieren en de opzet van de dierproef en welk keuzes daarbij zijn gemaakt.

Replacement
The biological coatings we would like to test are thought to stimulate bone formation due to their immunogenic properties.
The complexity of the bone environment and the interaction between immune and bone cells are difficult to mimick in vitro. Although we and others try to predict their effects on bone cells in vitro [1], their actual effects on bone tissue in vivo effects seem to be very different [3,4]. However, in vitro studies are still performed where possible, for instance to determine the optimal coating methods for the in vivo studies. For this purpose, the differentiation of stem cells into bone cells can be tested on the different coated constructs in vitro.

Reduction

Although the spinal fusion model requires relatively large group sizes to demonstrate statistically significant differences, there are a number of ways by which the number of rabbits can be reduced.
First, ectopic implantations can be performed in parallel so that multiple research questions can be answered within one experiment. The ectopic screening model allows for a large number of implants to be studied. We have previously used up to 18 samples in rabbits. Furthermore, the subcutaneous and

intramuscular locations can be easily studied in parallel.
Second, mechanistic studies are first performed in a screening setting ectopically. As the spinal fusion requires large group sizes, only the optimal conditions will be tested here. As such, 2-3 groups in total are sufficient.
Third, spinal fusion at two vertebral levels means that 2 treatments can be compared to the control in the same study.

Refinement

- Skin wounds will be sutured intracutaneously, using resorbable sutures
- Animals are housed together as much as possible
- Food is given ad libitum (pellets and hay)
- Animals are weighed weekly to monitor health
- Animals will be given 1 week to acclimatize to their new environment
- Eye ointment will be used to prevent dry eyes during surgery
- During and after surgery the animals will be placed on heat blankets
- Animals will receive adequate anaesthetics to prevent harm during surgery
- Analgesia medication will be administered untill 2 days after surgery to prevent harm post-operatively
- Daily scoring of the animals after the operations. Pain medication will be continued if necessary. For rabbits, this is a standard procedure after the OR

References:

1. Mo et al. Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors. BMC Cell biology. 2008.
2. Croes et al. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. Plos One. 2015.
3. Croes et al. Local inflammation and bone formation in a new translational animal model. In preparation.
4. Croes et al. Bacterial infection increases bone volume in a rabbit osteomyelitis model. Submitted.

Geef aan welke maatregelen zijn genomen om de kans op pijn, lijden of angst bij de dieren en de kans op nadelige milieueffecten tot een minimum te beperken.

The animals will have at least 1 week to acclimatize to the new environment before surgery. Animals will be given food and water ad libitum. The rabbits will be monitored for the respiratory and heart function by the designated biotechnician. The animals will be placed on heat blankets postoperatively. The animals will be returned to routine housing after they have recovered from anesthesia. For specific information see 'refinement'.

The animals will be housed conform the standards of the GDL Utrecht. They will be housed in groups of two as much as possible. This has been done for female New Zealand White rabbits and male Fischer rats in the past, usually without problems. In cases when animals show signs of agression towards each other, the animals will be housed individually to prevent harm.
The animals are weighed weekly by the animal care takers or researcher and monitored weekly (written and signed off in the working plan). This observation frequency is higher (once daily) after the surgeries. When weight loss is suspected later in the experiment, the animals will be monitored for the humane

endpoints and weighed daily.

Herhaling en duplicering

E. Herhaling

Geef aan hoe is nagegaan of deze dierproeven niet al eerder zijn uitgevoerd. Indien van toepassing geef aan waarom duplicatie noodzakelijk is.

n.v.t.

Huisvesting en verzorging

F. Huisvesting en verzorging

Worden de dieren anders dan volgens de eisen in bijlage III van de richtlijn 2010/63/EU gehuisvest en/of verzorgd?

☒ Nee

☐ Ja > Geef, indien dit kan resulteren in nadelige effecten op het dierenwelzijn, aan op welke wijze de dieren worden gehuisvest en verzorgd en motiveer de keuze om af te wijken van de eisen in bovengenoemde bijlage III.

G. Plaats waar de dieren worden gehuisvest

Worden de dierproeven geheel of gedeeltelijk uitgevoerd bij een inrichting die niet onder de rechtstreekse verantwoordelijkheid van een instellingsvergunninghouder Wod valt?

☒ Nee > Ga verder met vraag H.

☐ Ja > Geef aan wat voor bedrijf of instelling dit betreft.

Waarom is hiervoor gekozen en hoe wordt een adequate huisvesting, verzorging en behandeling van de dieren gewaarborgd?

Ongeriefinschatting / humane eindpunten

H. Pijn en pijnbestrijding

Valt te voorzien dat er pijn kan optreden bij de dieren?

☐ Nee > Ga verder met vraag I.

☒ Ja > Worden in dat geval verdoving, pijnstilling en/of andere pijnverlichtingsmethoden toegepast?

☐ Nee > Motiveer dan waarom geen pijnverlichtingsmethoden worden toegepast.

☒ Ja

I. Overige aantasting van het welzijn en maatregelen

Welke eventuele andere vormen van welzijnsaantasting worden voorzien?

Cumulative moderate (matig) stress is estimated based on the following procedures:

-Operations: moderate stress. Due to anesthetics and ectopic implantation of samples and implantation next to the spinal column. Animals may experience pain in the dorsum after the operation. When autologous bone is used, the animals may experience pain in the hip bone from bone harvesting. Animals will have reduced food intake and have weight loss due to the operations.
-Daily scoring of animals after the operation: mild stress due to handling.
-Injection of pain medication subcutaneously: mild stress due to handling and injection.
-Injection of fluorochrome markers subcutaneously (max. 3 times depending on duration of experiment): moderate stress caused by handling and local irritation of the skin (procedure causes mild stress, but considered as moderate as it is repeated).
-In vivo micro-CT scans (max.3 times dependening on duration of experiment): moderate moderate stress due to handling and anesthetic induction (procedure causes mild stress, but is considered as moderate as it is repeated)
-Blood harvest (rabbits, max. 5 times depending on studied factor and duration of experiment): moderate due to handling and ear irritation (procedure causes mild stress, but it is considered as moderate as it is repeated).
Euthanasia: mild stress due to handling.

Geef aan wat de mogelijke oorzaken hiervan zijn.

All described measurements are needed to create the least harm for the animals and most secure outcomes for this project.

Beschrijf welke maatregelen worden genomen om deze schadelijke effecten te voorkomen of waar mogelijk te minimaliseren.

1. At least 1 week to acclimatize to the new environment before surgery
2. Adequate use of injection anesthetics during the implantation procedure
3. The depth of narcosis (twitching/movement) can also be observed by the researcher performing the procedure. In the case of rabbits, the respiratory and heart function will be monitored by the biotechnician assisting with the anesthetics during the OR.
4. Adequate observation of vital signs of the animals post-operatively. Scoring of the animals daily after operations by the animal caretakers and the researcher.
5. For euthanasia, one of the methods listed in Appendix IV of directive 2010/63/EU is used
6. Pain medication, starting before the operation, and continued for two days as standard. If there is an indication, pain medication will be extended.

J. Humane eindpunten

Valt te voorzien dat zich bij deze dierproef omstandigheden voordoen waarbij het toepassen van humane eindpunten geïndiceerd is om verder lijden van de dieren te voorkomen?

☐ Nee > Ga verder met vraag K.

☒ Ja > Geef aan welke criteria hierbij worden gehanteerd.

- The animals will be euthanized in case one or more of the following humane endpoints are observed:
- Weight loss. When weight loss is suspected (reduced food intake), the animals will be weighed daily to monitor welfare
- Severe lethargy
- Severe tachycardia
- Severe tremor
- Loss of the animals' ability to walk and feed themselves
- Persistent infection of wounds.

-

Welk percentage van de dieren loopt kans deze criteria te halen?

Rats: not expected.
Rabbits: very unlikely, max. 5%.

K. Classificatie van ongerief

Geef aan hoe in het licht van alle hierboven beschreven negatieve effecten het cumulatief ongerief wordt geclassificeerd in termen van '*terminaal'*, *'licht'*, *'matig'* of *'ernstig'* ongerief.

The procedure is classified as moderate (matig).

Einde experiment

L. Wijze van doden

Worden de dieren als onderdeel van het experiment of na afloop van het experiment gedood?

☐ Nee > Ga verder met de ondertekening.

☒ Ja > Geef aan waarom het doden van dieren als eindpunt essentieel is voor deze proef.

The animals need to be euthanized to harvest the implants (ectopic model) for histology.

Wordt er een methode(n) van doden uit bijlage IV van richtlijn 2010/63/EU toegepast?

☐ Nee > Beschrijf de euthanasiemethode en onderbouw de keuze hiervoor.

☒ Ja

A. Algemene gegevens over de procedure

1. Aanvraagnummer : 2015.II.548.037
2. Titel van het project : Slimme coatings voor orthopedische implantaten
3. Titel van de NTS : Slimme coatings voor orthopedische implantaten

4. Type aanvraag:
   - ☒ nieuwe aanvraag projectvergunning
   - ☐ wijziging van vergunning met nummer :

5. Contactgegevens DEC

Naam DEC	: DEC Utrecht
Telefoonnummer contactpersoon	: 088 – 75 59 247
Emailadres contactpersoon	: dec-utrecht@umcutrecht.nl

6. Adviestraject (data dd-mm-jjjj):
   - ☒ ontvangen door DEC: 09-10-2015
   - ☐ aanvraag compleet:
   - ☒ in vergadering besproken: 21-10-2015 en 16-12-2015
   - ☒ anderszins behandeld: per mail: 24-12-2015
   - ☒ termijnonderbrekingen van / tot : 23-10-2015 tot 04-12-2015
     18-12-2015 tot 23-12-2015
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ☒ advies aan CCD: 18-02-2016

7. Eventueel horen van aanvrager
   - Datum:
   - Plaats:
   - Aantal aanwezige DEC-leden:
   - Aanwezige (namens) aanvrager:
   - Strekking van de vraag / vragen:
   - Strekking van het (de) antwoord(en):
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag:

8. Correspondentie met de aanvrager
   - Datum: 23-10-2015
   - Strekking van de vragen:

   Projectvoorstel:
   - 3.1 Achtergrond

De DEC is verbaasd over het feit dat referenties ontbreken naar het werk van uw eigen groep gezien uw werk de afgelopen jaren in het konijnenmodel. Wat is hiervoor de reden?

- 3.2 Doel
  Belangrijk is dat u een en ander dusdanig formuleert dat er sprake is van 1 onderzoeksdoel met meerdere subdoelen. Niet 3 doelen dus. Graag aanpassen.

- 3.2 en 3.3
  Op verschillende plaatsen in de tekst noemt u 'biofunctional molecules', 'bone-stimulating' factors etc. zonder dat u deze benoemt. Dit moet beslist wel gedaan worden, zeker ook in de bijlagen. Graag ook aangeven dat u in het kader van dit project hier geen onderzoek aan doet, maar vaart op eigen gegevens uit andere projecten.

- 3.4.1
  U schrijft: 'Finally, the rabbits immune system is more comparable to humans allowing for lower bacterial doses to be used'. Dit zult u anders moeten formuleren.

- 3.4.3.
- Welke S. aureus stam gebruikt u?
- Phase 3: De zin 'In vivo studys will be performed etc.' is onbegrijpelijk, graag anders formuleren.
- Phase 4: Waarop is de 1x10^5 CPU S. aureus bij het konijn gebaseerd? Graag referentie geven.

<u>Bijlage algemeen:</u>
Graag voor alle studies motiveren waarom u extra dieren nodig heeft.

<u>Bijlage 1:</u>
- Pagina 5: Pilot studie in ratten. Beter uitleggen wat de read-out is, en wat te verstaan onder een 'reproducible infection of the implant'.
- Een keer de afkorting NT verklaren.
- Pagina 6: Groeifactoren in antibiotica benoemen.

<u>B. De dieren</u>
- Pagina 7: Verwijderen zin: 'As an advantage, we have experienced…', want waarom neemt u dan geen Fisher ratten?
- Zijn de konijnen niet te jong? Is het groeiproces niet van invloed op uw data? Advies: volwassen dieren gebruiken.

<u>Bijlage 2:</u>
- Pagina 3: Ectopische implantaties: Wat is de ratio van de 2 staps implantatie?

- Pagina 5: Graag beter de extra dieren motiveren. Het is de DEC niet duidelijk waarom er geen extra dieren aangevraagd worden voor de spinal fusion experimenten.

B. De dieren

- U schrijft hier dat u werkt met Fischer ratten. Dit is niet consistent met bijlage 1.
- Ook hier weer de leeftijd van de konijnen motiveren, wellicht volwassen konijnen gebruiken?

Niet Technische Samenvatting

- Is de looptijd van 5 jaar wel reëel? Indien van toepassing gaarne aanpassen.

- Datum antwoord: 04-12-2015
- Strekking van het (de) antwoord(en):

Projectvoorstel:

- 3.1 Achtergrond
  We hebben een aantal referenties toegevoegd. Daarnaast refereren we naar eigen werk dat nog niet gepubliceerd is, maar dat hebben we voor de zekerheid bewaard voor de bijlage 2 in verband met de confidentiality.

- 3.2 Doel
  Akkoord, dit is aangepast.

- 3.2 en 3.3
  We hebben de tekst gewijzigd zoals u suggereert. We hebben daarnaast de term 'bacterial killing element' overal vervangen door 'inorganic nanoparticles', omdat dit specifieker is.

- 3.4.1
  Dit hebben we gewijzigd, hetzelfde geldt voor dezelfde formulering in de bijlagen.

- 3.4.3
- Dit is waarschijnlijk ATCC 6538, omdat momenteel de in vitro experimenten hiermee gedaan worden. Dit hebben we er nu bij vermeld.
- Phase 3: Akkoord
- Phase 4: Binnen onze groep is een pilotstudie gedaan om de dosis S. aureus te bepalen (DEC2012.III.04.037-C2). Deze data zijn niet gepubliceerd. Hierbij zijn bacteriële doses variërend van 10^2 tot 10^6 CFU getest. Bij 10^5 CFU was bij alle konijnen na een maand een infectie te zien. Bij 10^6 CFU was er sprake van een hematogene infectie.

Bijlage algemeen:

We hebben het nu bij elke studie vermeld.

Bijlage 1:

- Pagina 5: We zijn op zoek naar de minimale dosis S. aureus waarvoor een chronische infectie wordt waargenomen, gedefinieerd als de aanwezigheid van bacteriën in het bot na 4 weken. We spreken van een reproduceerbaar effect als een actieve infectie wordt aangetoond in alle drie de dieren van de groep.
- Pagina 6: Akkoord

B. De Dieren

- Pagina 7: Deze zin hebben we verwijderd, voor het infectiemodel worden inderdaad Sprague Dawley ratten gebruikt. Voor deze ratten geldt echter ook dat ze sociaal gehuisvest kunnen worden.
- We hebben in het verleden altijd adolescente konijnen gebruikt. Uit de literatuur kunnen we niet opmaken dat volwassen konijnen beter zouden zijn. Voor ons biedt een kleiner konijn het voordeel dat deze in het micro-CT-apparaat past waardoor in vivo scans gemaakt kunnen worden. Deze overwegingen hebben we nu vermeld in de aanvraag.

Bijlage 2:

- Pagina 3: Het is ons onduidelijk wat precies de vraag is, maar wellicht gaat het om de duur van de twee operaties. De eerste operatie (implantatie van de spacers) duurt ongeveer 1 uur. De tweede operatie (implantatie van de geladen constructen) duurt ongeveer 1.5 uur. Door eerst een spacer te implanteren, wordt een holte gecreëerd, omgeven door een membraan. Dit membraan zorgt voor een meer gelokaliseerde ontstekingsreactie, omdat het de diffusie en het lekken van ontstekingsfactoren naar het omliggende weefsel moet beperken. Dit membraan is deels gekarakteriseerd, maar we willen additionele experimenten doen om de eigenschappen van het membraan in kaart te brengen. We hebben bij de omschrijving van het diermodel nu wat meer details gegeven en een bron toegevoegd.
- Pagina 5: We hebben deze zin weggehaald om verwarring te vermijden. We vragen voor de spinal fusion model wel degelijk extra dieren aan rekening houdend met 5-6% uitval van dieren. Alle dieren ondergaan beide procedures.

B. De dieren

- Voor het infectiemodel (bijlage 1) gebruiken we Sprague-Dawley ratten, omdat deze het meest beschreven worden in de literatuur. Voor de wervelfusie en ectopische modellen gebruiken we Fischer ratten. Voor botvorming studies hebben we veel ervaring met Fischer ratten, met name bij het gebruik van calcium fosfaten en BMP-2 groeifactor. De hoeveelheid botvorming bij de gekozen tijdspunten kan daarom goed voorspeld worden.
- Hoewel de spinal fusion rate misschien niet veel wordt beïnvloed door de leeftijd, zijn we het ermee eens dat het verstandiger is om volwassen konijnen te gebruiken. In de literatuur worden met name konijnen van 6 maanden of ouder gebruikt. Dit weegt naar onze mening op tegen het criterium dat de konijnen in vivo gescand kunnen worden in de micro-CT

apparaat. Met zulke grote konijnen moet het namelijk nog blijken of dit inderdaad praktisch haalbaar is. Dit is aangepast

Niet Technische Samenvatting

- Een looptijd van 5 jaar lijkt ons reëel. Er zijn technieken die nog ontwikkeld moeten worden en vervolgens in vitro getest moeten worden. We hebben dit benadrukt in het projectvoorstel (3.2).

- Datum: 18-12-2015
- Strekking van de vragen:

Bijlage 2

- A. Experimentele aanpak en primaire uitkomstparameters: Het is de DEC niet direct duidelijk waarop de uitval met betrekking tot de spinal fusion gebaseerd is. Graag toelichten.
- A. Experimentele aanpak en primaire uitkomstparameters: De DEC adviseert het argument met betrekking tot anesthesie te verwijderen, omdat dit tegenwoordig niet meer voorkomt.
- A. Experimentele aanpak en primaire uitkomstparameters: De motivatie voor het extra aantal dieren graag overleggen en aanpassen in samenspraak met de IvD.

- Datum antwoord: 23-12-2015
- Strekking van de antwoorden:

Bijlage 2

- A. Experimentele aanpak en primaire uitkomstparameters: Voor de konijnen beschrijft men in de literatuur voornamelijk uitval als gevolg van infecties of complicaties gerelateerd aan de isolatie van autoloog bot uit de bekkenkam (e.g. bloedverlies, verlamming). In onderstaande referenties beschrijft men gemiddeld gezien een uitval van 8%. Omdat maar een deel van de konijnen autologe bottransplantatie zal ondergaan in onze studies (ongeveer één derde), denken we dat een gemiddelde uitval van 5% gezien over alle konijnen een realistische schatting is. Voor de ratten hebben we de extra dieren verwijderd uit de aanvraag voor de spinal fusion gedeelte, aangezien deze complicaties minder een rol lijken te spelen hier. Bij de ratten zal geen autoloog bot geïsoleerd worden. Deze motivatie hebben we nu in de bijlage vermeld.

- A. Experimentele aanpak en primaire uitkomstparameters: We hebben dit argument weggehaald, we zijn het ermee eens dat dit niet de belangrijkste reden van uitval is.
- A Experimentele aanpak en primaire uitkomstparameters: Zie hierboven de uitleg voor bijlage 2 (spinal fusion). Voor bijlage 1 (infectie model) hebben we in samenspraak met de IVD het aantal extra dieren per experiment aangepast. Op basis van de literatuur hebben we het percentage extra dieren verhoogd van 5% naar 10%. Hoewel de exacte reden van uitval van

de ratten in deze papers niet wordt genoemd, zegt onze ervaring in konijnen dat sepsis na S. aureus infectie (2013.III.11.083 60 dieren) een belangrijke reden hiervoor is. Deze motivatie hebben we nu in de bijlage vermeld. Voor de pilotstudie is het percentage uitval verhoogd naar 20%, omdat overdosering met S. aureus hier nog een rol kan spelen.

- De antwoorden hebben geleid tot aanpassing van de aanvraag: Ja

9. Eventuele adviezen door experts (niet lid van de DEC)
   - Aard expertise:
   - Deskundigheid expert:
   - Datum verzoek:
   - Strekking van het verzoek:
   - Datum expert advies:
   - Expert advies:

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.
4. Er zijn geen DEC-leden betrokken bij het betreffende project.

C. Beoordeling (inhoud):

1. Het project is:
   - ☒ uit wetenschappelijk oogpunt verantwoord.
   - ☐ uit onderwijskundig oogpunt verantwoord.
   - ☐ uit het oogpunt van productiedoeleinden verantwoord.
   - ☐ wettelijk vereist.

2. De in de aanvraag aangekruiste doelcategorieën zijn in overeenstemming met de hoofddoelstellingen.

3. De DEC onderschrijft het belang van de doelstelling. In 10% van de gevallen waarbij een botimplantaat in kleine botdefecten geplaatst wordt is de ingreep niet succesvol, doordat bacteriën – ondanks adequate voorzorgsmaatregelen – tijdens de operatie aan het implantaat hechten en het vervolgens infecteren, en/of doordat het implantaat onvoldoende integreert in het omliggende botweefsel. De aanvrager wil met behulp van een model voor implantaatinfectie (bijlage 1) onderzoeken of het mogelijk is om multifunctionele coatings te ontwikkelen die zowel het hechten en uitgroeien van bacteriën voorkomen, alsook botgroei stimuleren. Daarnaast wil de aanvrager met behulp van een *spinal fusion* model (bijlage 2) onderzoeken of de coatings ook geschikt zijn voor toepassing bij grote botdefecten. Grote botdefecten kunnen alleen genezen met behulp van uitgebreide *de novo* botvorming, en de gouden standaard om

dit te bewerkstelligen is het plaatsen van autoloog bot. *Spinal fusion* is een erkend model voor onderzoek naar stimulatie van *de novo* botvorming, en een veel voorkomende indicatie voor transplantatie van autoloog bot bij patiënten. De zogenaamde 'slimme coatings' die met behulp van dit project ontwikkeld worden kunnen mogelijk het succes van botimplantaties vergroten, en de negatieve gevolgen van infectie, onvoldoende integratie van een implantaat en het oogsten van autoloog bot (zoals pijn, invaliditeit en heroperaties) voorkomen. Het belang van de doelstelling wordt door de DEC ingeschat als substantieel.

4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. Het project is opgedeeld in logisch op elkaar volgende fasen met duidelijke go/no-go momenten. In bijlage 1 worden verschillende experimenten uitgevoerd in een model voor implantaatinfectie in ratten en konijnen, waarbij van verschillende coatings de antibacteriële effecten en stimulatie van botgroei richting een aangebracht implantaat bestudeerd kunnen worden. Eerst zal het reeds in konijnen gevalideerde model voor toepassing in ratten geoptimaliseerd worden (fase I). Vervolgens wordt de werkzaamheid van coatings die in *in vitro* experimenten veelbelovende resultaten lieten zien in ratten onderzocht (fase II en III). (NB de *in vitro* experimenten zijn geen onderdeel van deze projectaanvraag). In fase II wordt de werkzaamheid van coatings met antibacteriële componenten in ratten bestudeerd. De experimenten van fase III – waarin de componenten met een antibacterieel effect gecombineerd worden met groeifactoren – worden alleen uitgevoerd, wanneer *in vitro* experimenten hebben laten zien dat de combinatie van deze componenten effectiever is dan de antibacteriële componenten alleen. Alleen coatings die in fase II en/of III werkzaam bleken te zijn worden vervolgens ook in konijnen onderzocht (fase IV). In bijlage 2 worden verschillende experimenten uitgevoerd met behulp van een *spinal fusion* model in ratten en konijnen, waarbij het vermogen van coatings om *de novo* botvorming te stimuleren bestudeerd kan worden. In eerste instantie wordt de werkzaamheid van de coatings in ratten onderzocht (fase V). Ook hier geldt dat alleen coatings die in ratten werkzaam bleken te zijn vervolgens ook in konijnen onderzocht worden (fase VI). De DEC is ervan overtuigd dat de aanvrager over voldoende expertise en voorzieningen beschikt om de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn te realiseren.

5. Er is geen sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren:
   - ☐ Bedreigde diersoort(en) (10e lid 4)
   - ☐ Niet-menselijke primaten (10e)
   - ☐ Dieren in/uit het wild (10f)
   - ☐ Gefokt voor dierproeven (11)
   - ☐ Zwerfdieren (10h)
   - ☐ Hergebruik (1e lid 2)
   - ☐ Huisvesting en verzorging
   - ☐ Locatie: instelling vergunninghouder (10g)

6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. Bij alle dieren van de bijlagen 1 en 2 zullen een of meerdere implantaten aangebracht worden. Het ongerief ten gevolge van de daarvoor noodzakelijke chirurgische ingrepen wordt ingeschat als matig. Andere experimentele ingrepen die uitgevoerd zullen worden (zoals fluorochroom injecties, bloedafnames, oogsten van autoloog bot en herhaaldelijk bijkomen uit anesthesie ten behoeve van imaging) leiden ook tot matig ongerief. In bijlage 1 wordt bij alle implantaten een lokale infectie geïnduceerd. Men houdt er rekening mee dat deze infectie ook systemische effecten kan hebben, en dat maximaal 10% van de ratten (en maximaal 20% in de pilotstudie) en 5% van de konijnen ten gevolge hiervan het humane eindpunt zullen bereiken. Bij de experimenten beschreven in bijlage 2 zullen complicaties na het oogsten van autoloog bot bij maximaal 5% van de konijnen leiden tot het bereiken van het humane eindpunt. Aangezien bij de ratten geen autoloog bot zal worden geoogst wordt niet verwacht dat een of meerdere ratten het humane eindpunt zullen bereiken.

7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen vervangen. Antibacteriële en botgroeistimulerende effecten van de te onderzoeken coatings worden in eerste instantie met behulp van *in vitro* experimenten onderzocht. Vervolgens worden de effecten van de meest veelbelovende coatings in een *in vivo* setting getest, omdat eerder uitgevoerde experimenten hebben uitgewezen dat resultaten van *in vitro* experimenten onvoldoende voorspellende waarde hebben voor effecten die *in vivo* waargenomen kunnen worden. Interacties tussen componenten van de coatings, immuuncellen en botweefsel liggen ten grondslag aan de antibacteriële en botgroei-stimulerende effecten van de coatings. Deze interacties zijn dusdanig complex, dat ze niet in hun volledigheid *in vitro* of *in silico* nagebootst kunnen worden. Het is bijvoorbeeld niet mogelijk om botvorming *in vitro* te bewerkstelligen. Wel is het mogelijk om te onderzoeken in hoeverre bepaalde componenten van de coatings in staat zijn om differentiatie van stamcellen tot botcellen te stimuleren. Dergelijk voorbereidend werk wordt in de voorliggende projectaanvraag *in vitro* uitgevoerd, voordat men overgaat tot de beschreven dierexperimenten. In verband met de opzet en de risico's van het onderzoek (zoals het aanbrengen van geïnfecteerde implantaten) en de aard van de benodigde gegevens (zoals morfologisch onderzoek van implantaten en omliggend weefsel) is het niet mogelijk om het onderzoek in mensen uit te voeren.

8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de vermindering van dierproeven. Het optimale aantal dieren per groep wordt voorafgaand aan de experimenten bepaald door powerberekeningen uit te voeren, waar mogelijk met behulp van gegevens uit eerder uitgevoerde *in vitro* en *in vivo* experimenten. Daarbij wordt rekening gehouden met het feit dat niet alle vergelijkingen die mogelijk zijn, ook daadwerkelijk gemaakt zullen worden. Zo wordt voorkomen dat de alpha teveel gecorrigeerd en daarmee de groepsgrootte onnodig groot wordt. Eerder uitgevoerde experimenten hebben uitgewezen dat het mogelijk is om in een dier meerdere ectopische implantaten en orthotope implantaten aan te brengen. De

implantaten beïnvloeden elkaar niet, en het plaatsen van meerdere implantaten heeft geen negatieve gevolgen voor het dier. Op deze wijze kan het aantal dieren dat nodig is voor de experimenten in bijlage 2 op verantwoorde wijze beperkt worden. Het maximale aantal te gebruiken dieren is realistisch ingeschat.

9. Het project is in overeenstemming met de vereiste van de verfijning van dierproeven en het project is zo opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd.
   De onderzoeksgroep heeft veel ervaring met de beschreven diermodellen en bijbehorende analysemethoden. Het welzijn van de dieren wordt gedurende de experimenten nauwlettend in de gaten gehouden. Om ongerief zoveel mogelijk te beperken worden adequate – en op de betreffende ingreep/handeling afgestemde – analgesie- en anesthesieprotocollen toegepast. Wanneer na afloop van een chirurgische ingreep blijkt dat de dieren onvoldoende herstellen, dan worden ondersteunende maatregelen getroffen. Mocht een dier desondanks in conditie verslechteren en het humane eindpunt bereiken, dan wordt het geëuthanaseerd. Op deze wijze wordt getracht te voorkomen dat dieren meer dan matig ongerief ervaren.
   In beide modellen worden de experimenten in eerste instantie in ratten uitgevoerd, omdat voor deze diersoort veel analytische hulpmiddelen beschikbaar zijn, waardoor een mechanistisch onderzoek naar de werkzaamheid van coatings mogelijk is. De coatings die de beste resultaten opleveren zullen vervolgens onderzocht worden in konijnen, omdat het konijn qua immuunsysteem meer overeenkomsten vertoont met de mens. Ook de grootte van de implantaten die aangebracht kunnen worden en de lagere dosis *Staphylococcus aureus* die een chronische infectie induceert geven een betere benadering van de situatie in de mens.
   Daarnaast is het mogelijk om in het *spinal fusion* model in konijnen de werkzaamheid van gecoate implantaten te vergelijken met de gouden standaard die in de kliniek toegepast wordt: de transplantatie van autoloog bot. Een dergelijke ingreep is bij ratten technisch lastig uitvoerbaar in verband met de grootte van de dieren.
   In beide bijlagen zullen alleen mannelijke ratten worden ingezet, omdat de implantaten die bij vrouwtjes aangebracht kunnen worden een kleiner oppervlak hebben. Daardoor is het technisch lastig uitvoerbaar om de te onderzoeken coatings aan te brengen. In beide bijlagen zullen alleen vrouwelijke konijnen ingezet worden, omdat dit een goede vergelijking met resultaten uit eerdere experimenten mogelijk maakt. Bijkomend voordeel is dat vrouwelijke konijnen in groepsverband gehuisvest kunnen worden. Daarnaast is bekend dat het botmetabolisme in ratten en de *fusion rate* in konijnen tussen mannelijke en vrouwelijke dieren verschilt, waardoor het gebruik van beide geslachten zou leiden tot een aanzienlijke toename van de variantie en de bijbehorende benodigde groepsgroottes.

10. De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd.

D. Ethische afweging

Op grond van de onder C genoemde overwegingen is de DEC van mening dat het belang van de doelstelling – het ontwikkelen van multifunctionele coatings, die zowel het hechten en uitgroeien van bacteriën op implantaten voorkomen, alsook botgroei stimuleren – substantieel is. Het projectvoorstel is vanuit wetenschappelijk oogpunt verantwoord en de vertaling van onderzoeksresultaten naar de mens wordt mogelijk geacht. De DEC is van mening dat de juiste onderzoeksstrategie gekozen is, en dat de verschillende diermodellen en experimenten noodzakelijk zijn voor en leiden tot het bereiken van de doelstelling. Het gelijktijdig werken in de twee beschreven diermodellen acht de DEC een sterk punt, omdat het bestuderen van de twee verschillende vormen van botimplantatie die in de kliniek voorkomen een vollediger beeld geeft van de effecten die de coatings kunnen bewerkstelligen. De DEC is ervan overtuigd dat bij de dierproeven adequaat invulling gegeven zal worden aan de vereisten op het gebied van vervanging, vermindering en verfijning van dierproeven, mede doordat de onderzoeksgroep veel ervaring heeft met de beschreven diermodellen en het project is opgesplitst in logisch op elkaar volgende fasen met duidelijke criteria voor go/no-go beslissingen. Dit alles brengt de DEC tot het oordeel dat het belang van het voorliggende onderzoek opweegt tegen het matige ongerief dat de dieren zullen ondervinden, en acht het gebruik van de dieren voor dit project ethisch aanvaardbaar.

E. Advies

1. Advies aan de CCD

   ☐ De DEC adviseert de vergunning niet te verlenen vanwege:

   ☐ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden

   ☒ De DEC adviseert de vergunning te verlenen.

2. Het uitgebrachte advies is gebaseerd op consensus.

Dierexperimentencommissie Utrecht

7

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

UMC Utrecht
[redacted]
Postbus 12007
3501 AA UTRECHT

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD115002016445

Bijlagen
2

Datum 24 februari 2016
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte [redacted]
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 23 februari 2016.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD115002016445. Gebruik dit nummer wanneer u contact met de CCD opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	11500
Naam instelling of organisatie:	UMC Utrecht
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	30244197
Postbus:	12007
Postcode en plaats:	3501 AA UTRECHT
IBAN:	NL27INGB0000425267
Tenaamstelling van het rekeningnummer:	Universiteit Utrecht

Gegevens verantwoordelijke onderzoeker	
Naam:	[redacted]
Functie:	PhD Onderzoeker
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Gegevens plaatsvervangende verantwoordelijke onderzoeker

Naam:	[redacted]
Functie:	[redacted]
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Gegevens verantwoordelijke uitvoering proces

Naam:	[redacted]
Functie:	[redacted]
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Over uw aanvraag

Wat voor aanvraag doet u?	[x] Nieuwe aanvraag [ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn [ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Over uw project

Geplande startdatum:	1 januari 2016
Geplande einddatum:	1 januari 2021
Titel project:	Smart coatings for orthopedic implants
Titel niet-technische samenvatting:	Slimme coatings voor orthopedische implantaten
Naam DEC:	DEC Utrecht
Postadres DEC:	Postbus 85500 3508 GA Utrecht
E-mailadres DEC:	dec.utrecht@umcutrecht.nl

Betaalgegevens

De leges bedragen:	€ 741,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting
Overige bijlagen:	[x] DEC-advies

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Utrecht
Datum:	19 februari 2016

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

UMC Utrecht
[redacted]
Postbus 80.011
3508 TA UTRECHT

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD115002016445
Bijlagen
2

Datum 24 februari 2016
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 24 februari 2016
Vervaldatum: 25 maart 2016
Factuurnummer: 16700445
Ordernummer: CB. 841910.3.01.011

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD115002016445	€ 741,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL28RBOS 056.99.96.066 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 20401, 2500 EK te 's Gravenhage.

[redacted]

Van: Info-zbo
Verzonden: woensdag 23 maart 2016 9:41
Aan: [redacted]
CC: [redacted]
Onderwerp: aanvraag AVD115002016445

Geachte [redacted]

U heeft bij de CCD een aanvraag voor vergunning ingediend. Het betreft uw project "Smart coatings for orthopedic implants" met aanvraag nummer AVD115002016445. In de bijlagen dierproeven is het aantal dieren niet consistent weergegeven. Wij willen u vragen dit met elkaar in overeenstemming te brengen.
In bijlage 3.4.4.1 beschrijft u onder A. een totaal van 66 ratten en 70 konijnen en onder B. 70 ratten en 70 konijnen.
In bijlage 3.4.4.2 beschrijft u onder A. een totaal van 294 ratten en 176 konijnen en onder B. 279 ratten en 176 konijnen.

Wij gaan ervan uit dat de onder A. genoemde aantallen correct zijn omdat dit ook overeen komt met de NTS, wanneer dit niet zo is , dan verzoeken wij u om ook de NTS aan te passen.

Met vriendelijke groet, [redacted]

Centrale Commissie Dierproeven
www.centralecommissiedierproeven.nl
.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl (let op: nieuw emailadres!)

Instantie voor Dierenwelzijn Utrecht

bezoekadres
Bolognalaan 50
3584 CJ Utrecht

postadres
Postbus 12007
3501 AA Utrecht

T (030) 253 15 69
info@ivd-utrecht.nl
www.ivd-utrecht.nl

Centrale Commissie Dierproeven
Postbus 20401
2500 EK s-GRAVENHAGE

uw kenmerk
ons kenmerk

datum 29 maart 2016
onderwerp Antwoorden AVD115002015445

Mijne Dames en Heren,

Naar aanleiding van uw mail d.d. 23 maart 2013, zend ik u bijgaand de gecorrigeerde bijlagen van bovenstaand project. Uw veronderstelling dat de aantallen on der A correct waren, is juist.

Met vriendelijke groet

[redacted]

Bijlage
Beschrijving dierproeven

- Deze bijlage voegt u bij uw projectvoorstel dierproeven.
- Per type dierproef moet u deze bijlage invullen en toevoegen.
- Meer informatie vindt u op de website www.zbo-ccd.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Vul uw deelnemernummer van de NVWA in.	11500
1.2	Vul de naam van de instelling of organisatie in.	UMC Utrecht
1.3	Vul het volgnummer en het type dierproef in. *Gebruik de volgnummers van vraag 3.4.4 van het format Projectvoorstel.*	Volgnummer: 1 Type dierproef: Implant infection model in rats and rabbits

2 Beschrijving dierproeven

A. Experimentele aanpak en primaire uitkomstparameters

Beschrijf de keuze van de experimentele aanpak en de primaire uitkomstparameters.

We would like to use a rat and rabbit model of an implant-related infection [1]. In this model, a metal implant is inserted in the tibiae of the animals while it is in the meantime inoculated with a specific dose of bacteria [2]. We will use S. aureus to induce a chronic infection, as this is a clinically relevant strain which is often associated with biofilm formation [3]. As we are using S. aureus ATCC 6538 for our in vitro studies, we will likely also use this strain for the animal studies. A pilot study will be performed in the rat model to determine the minimal dose of S. aureus needed to induce a chronic infection. For the rabbit model, such a pilot has already been performed within our group. As such, a dose of 10^5 CFU will be used. After several weeks (4 weeks normally, and 8 weeks when long-term effects are studied) [1] , the number of bacteria can be quantified as a measure of bacterial killing (bacterial culture and

histology). Furthermore, bone formation around the implant is quantified to determine the bone-implant integration (micro-CT imaging and histology) [2].

The bacterial-killing activity and bone-stimulating activity of our implant coatings will be comapared to untreated control implant. In our experiments, we would like to test titanium nanotubes [4], inorganic nanoparticles (e.g. zinc and silver) [5, 6], and layer-by-layer coatings [7] for their bacterial-killing and bone-stimulating activity. Growth factors such as the bone morphogenetic proteins can be used together with the inorganic nanoparticles to promote bone formation in the layer-by-layer coatings.

We will perform initial studies in rats, which have shown to be a very suitable animal model for this purpose due to their size allowing implant placement, easy handling, and the establishment of chronic infection with clinically-relevant bacterial strains [1]. When successful coatings are identified, we would also like to test their efficacy in the rabbit model. The rabbit model, due to their more comparable immune system to humans, is superior to the rat model in a translational aspect [8,9]. Lower doses of bacteria can generally be used in rabbits than in rats, which are more comparable to the clinical situation [1]. For the same reason, in addition to bacterial-killing and bone-implant integration, the rabbit model is a superior model to study local and systemic markers of inflammation. Finally, larger-size implants can be tested which allow for more surface modification.

The different experiments can be summarised af follows:

- test coatings with inorganic nanoparticles (e.g. zinc and silver) in rats. The different elements will be incorporated into titanium nanotube coatings. To embed silver nanoparticles in titanium oxide nanotubes, two different concentrations (0.5 and 1.5 M) of AgNO3 will be used [5]. Also two different concentrations (0.015 and 0.030M) of Zn(NO3)2 will be used [6]. We are currently testing the in vitro bacterial-killing activity of these elements. The animals will be euthanized after 4 weeks.

- test the controlled release of the aforementioned nanoparticles in rats. For this purpose, a thin layer of thermo-sensitive hydrogel will be used in combination with the nanotubes. As we are also interested in the more long-lasting effects of these coatings, animals will be euthanized at two different time points (4 and 8 weeks).

-test a layer-by-layer designed coating with dual purpose, e.g. bacterial-killing and stimulating bone-implant integration in rats. These layers consist of antimicrobials (e.g. antibiotics like gentamicin)[10] and growth factors (e.g. bone morphogenetic proteins)[11]. Here, also two different concentration from each agent will be studied in the same rat model. These components will be tested alone, or in combination, to determine the coating with best bacterial-killing and bone-stimulating activity. We are currently still developing the technology to produce the layer-by-layer coatings. If this is succesful, in vitro release profile of layer-by-layer components will be determined. Depending on the release profile that can be obtained in vitro, the animals will be euthanized after either 4 or 8 weeks.

- test the most promising conditions in the rabbit model. The rabbit model is a more clinically relevant model because lower doses of S. aureus can be used [1]. Furthermore, larger-size implants can be tested which allow for more surface modification. The same analyses will be performed as in the rats. In addition however, blood samples are harvested to measure systemic markers of inflammation. Local signs of inflammation are quantified by histology and cytokine measurements on tissues harvested locally. In this study, the rabbits are euthanized at 4 and 8 weeks to also test the long-lasting effects of the coatings.

REFERENCES

[1] Reizner, JG Hunter, NT O'Malley, RD Southgate, EM Schwarz, SL Kates. A systematic review of animal models for Staphylococcus aureus osteomyelitis. Eur Cell Mater. 2014 Mar 25;27:196-212.
[2] Moojen DJ, Vogely HC, Fleer A, Nikkels PG, Higham PA, Verbout AJ, Castelein RM, Dhert WJ. Prophylaxis of infection and effects on osseointegration using a tobramycin-periapatite coating on titanium implants--an experimental study in the rabbit. J Orthop Res. 2009 Jun;27(6):710-6.
[3] Montanaro L, Speziale P, Campoccia D, Ravaioli S, Cangini I, Pietrocola G, Giannini S, Arciola CR. Scenery of Staphylococcus implant infections in orthopedics. Future Microbiol. 2011 Nov;6(11):1329-49
[4] Gao A, Hang R, Huang X, Zhao L, Zhang X, Wang L, et al. The effects of titania nanotubes with embedded silver oxide nanoparticles on bacteria and osteoblasts. Biomaterials 2014;35:4223-35.
[5] Zhao L, Wang H, Huo K, Cui L, Zhang W, Ni H, et al. Antibacterial nano-structured titania coating incorporated with silver nanoparticles. Biomaterials 2011;32:5706-16.
[6] Liu W, Su P, Chen S, Wang N, Ma Y, Liu Y, et al. Synthesis of TiO2 nanotubes with ZnO nanoparticles to achieve antibacterial properties and stem cell compatibility. Nanoscale 2014;6:9050-62.
[7] Necula BS, Fratila-Apachitei LE, Zaat SAJ, Apachitei I, Duszczyk J. In vitro antibacterial activity of porous TiO2–Ag composite layers against methicillin-resistant Staphylococcus aureus. Acta Biomaterialia 2009;5:3573-80
[8] Bosze, Z. and Houdebine, L.M. (2006) Application of rabbits in biomedical research: a review. World Rabbit Sci. 14:1-14.
[9] Dharmadhikari, A. and Nardell, E.A. (2008) What animal models teach humans about tuberculosis. Am. J. Respir. Cell Mol. Biol. 39:503-508.
[10] Cosgrove SE, Vigliani GA, Fowler VG Jr, Abrutyn E, Corey GR, Levine DP, Rupp ME, Chambers HF, Karchmer AW, Boucher HW. Initial low-dose gentamicin for Staphylococcus aureus bacteremia and endocarditis is nephrotoxic. Clin Infect Dis. 2009 Mar 15;48(6):713-21
[11] Bishop GB, Einhorn TA. Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery. Int Orthop. 2007 Dec; 31(6): 721–727.

Beschrijf de beoogde behandeling van de dieren (inclusief de aard, de frequentie en de duur van de behandelingen waaraan de dieren worden blootgesteld) en onderbouw de gekozen aanpak.

Rats:

Surgical procedure- the left hind leg is shaven and disinfected with povidone iodine. An incision is made to the anteromedial aspect of the tibia. To access the medullary cavity, a hole between the tibial plateau and the tibial tuberosity is drilled with a smooth stainless steel pin (K-wire) through the cancellous bone of the proximal metaphysic. The K-wire is inserted into the medullary cavity and pushed forward distally to create space in the cavity. After removal, a small volume of either saline of bacteria are injected into the medullary cavity by a microsyringe. After bacterial inoculation, the differently coated K-wires are inserted and the skin and fascia are sutured with resorbable sutures. The surgeries are estimated to take half an hour. In vivo scanning is performed with appropriate anesthetics (max. 3 times depending on the duration of the experiment). Fluorochrome markers are injected subcutaneously (max. three time points depending on the duration of the experiment) to determine the onset and development of bone formation. The animals are euthanized after 4 or 8 weeks depending on the coating which is tested.

Anesthetics- start and maintained with isoflurane/oxygen

Pain management-injection analgetics (usually buprenorphine with 12 hour interval), starting before surgery and continued after surgery. The protocol will be developed in consultation with the designated veterinarian. If there is any indication that the rats continue to suffer pain, the pain medication will be prolonged.

Antiseptic techniques- Surgery will be performed under aseptic conditions. The skin will be disinfected with povidone iodine. The surgeon will wear scrubs, sterile gloves, and a surgery mask/cap. Only autoclaved instruments will be used.

Postoperative care- The animals will be placed on heat blankets. The animals will be housed per two if possible. The animals will be scored daily for well-being in the first week, and weekly thereafter. The rats will receive special food if they have reduced food intake due to the surgery and the pain medication.

Infection measurements- In vivo scanning under isoflurane anesthetics is performed for a maximum of 3 times in total. Bacterial culture of bone and implant after euthanization is the primary outcome measure for bacterial killing. Local inflammation markers will be measured on tissue samples.

Bone formation measurements- Bone-implant contact as determined on histology and aforementioned in vivo scans. The incorporation of fluorchromes is assessed by fluorescence microscope. This will elucidate the onset and progression of bone formation around the implant.

Euthanasia protocol- One of the methods listed in appendix IV of directive 2010/63/EU.
The rats will be euthanized at 4 or 8 weeks after start of the experiment with an overdose of CO_2 inhalation.

Rabbits:

Surgical procedure- The left hind leg is shaven and disinfected with povidone iodine. The stifle joint is opened with an incision through the skin and the patellar tendon. A hole is drilled into the tibia through the cartilage layer with a hand drill. The bacteria are inoculated into the cavity in a small volume with a pipette. A coated titanium implant is press-fit into the cavity, flushed with the cartilage surface. The patellar tendon and the skin are closed with resorbable sutures. The surgeries are estimated to take half an hour. In vivo micro-CT scanning is performed with appropriate anesthetics similar to the surgery (up to 3 times). Fluorochrome markers are injected subcutaneously to determine the onset and progression of bone formation (max. at 3 different time points). Blood is drawn from the ear vein (at max. 5 time points) to measure systemic markers of inflammation. The animals are euthanized after 4 and 8 weeks.

Anethesia- Combination of injection analgetics/anesthetics (in consultation with the designated veterinarian).

Pain management- Injection analgetics (usually buprenorphine with 12 hour interval), starting before surgery and continued after surgery (in consultation with the designated veterinarian). If any signs of pain continue, pain medication will be prolonged.

Antiseptic techniques- Surgery will be performed under aseptic conditions. The skin will be disinfected with povidone iodine. The surgeon will wear scrubs, sterile gloves, sterile gown, and a surgery mask/cap. Only autoclaved instruments will be used.

Postoperative care- The animals will be placed on heat blankets. The animals will be housed per two if possible. The animals will be scored daily for well-being in the first week and weekly thereafter. The rabbits will receive special food (soaked in water) when food intake is decreased (e.g. by systemic inflammation and as a side effect of the pain medication).

Infection measurements- In vivo scanning (under injection analgetics/anesthetics) is performed for a maximum of 3 times in total. Bacterial culture of bone and implant after euthanization is the primary outcome measure for bacterial killing. Systemic markers of inflammation are measured on blood samples.

Bone formation measurements- Bone-implant contact as determined on histology and aforementioned in vivo scans. The incorporation of fluorochrome markes will be determine by fluorescence microscopy to assess the onset and progression of new bone formation.

Euthanasia protocol- One of the methods listed in appendix IV of directive 2010/63/EU.
The rabbits will be euthanized after 4 and 8 weeks after the start of the experiment (usually with an overdose of Pentobarbital i.v.). This will be done under general anesthetics and pain medication, similar to the protocol used during surgery.

Geef aan welke overwegingen en statistische methoden worden gebruikt om het aantal benodigde dieren tot een minimum te beperken.

The following section describes the minimal number of animals needed for each study. For each study, a percentage of animals are added to the total to account for a possible loss of animals during the experiment. The percentage loss of animals is based on literature (rat experiments) or on our own experience with the same model and inoculation dose (rabbit experiments). Based on literature, ahigher percentage loss is expected in rats (10%) compared to rabbits (5%). Furthermore, we expect a higher percentage loss in the pilot study performed in rats, due to a higher chance of hematogenous infection. A pilot study has already been performed for the rabbit model by our group in the past.

STUDY 1, pilot study in rats (24 rats)
A pilot study will be performed to determine the minimal dose of S. aureus needed to get a chronic infection of the implant [1]. Although these doses are also described in literature [1], different experimental setting (bacterial culture conditions, materials, housing conditions) may affect the results. Our goal is not to demonstrate statistical significance differences. We believe the chosen group sizes will allow for the identification of the optimal bacterial dose. The minimal dose for which all three rats show the presence of bacteria in the bone after 4 weeks will subsequently be used to test the efficacy of the coatings.
Due to the relative higher chance of hematogenous infection for the higher CFU doses in this pilot study, a 20% of rats (4 rats) are added to the total to account for possible loss of animals.

1) Neg control (N=2)
2) 10^2 CFU S. aureus (N=3)
3) 10^3 CFU S. aureus (N=3)
4) 10^4 CFU S. aureus (N=3)
5) 10^5 CFU S. aureus (N=3)
6) 10^6 CFU S. aureus (N=3)
7) 10^7 CFU S. aureus (N=3)

For the following experiments, we will make an estimation of the the expected difference of the means and coefficient of variance will be based on the in vitro observations: 50% expected difference of the means and 50% coefficient of variance in bacterial killing. We will perform a one way ANOVA's with a Bonferroni post hoc correction. The alpha will be adjusted for the multiple comparions of the experimental groups (specified next per individual experiment). A two sided test will be used. We will use the S. aureus dose as determined in the pilot study.

STUDY 2, the effect of inorganic nanoparticles (73 rats):

1) Untreated implant (N=11)
2) Implant with nanotubes (NT) (N=11)
3) Implant with NT + Silver (AgNO3, 0.5 M) (N=11)
4) Implant with NT + Silver (AgNO3, 1.5 M) (N=11)
5) Implant with NT + Zinc (Zn(NO3)2, 0.015 M) (N= 11)
6) Implant with NT + Zinc (Zn(NO3)2, 0.030 M) (N= 11)

The best performing concentration of each element (3/4 and 5/6) is compared to the NT control (group 2).
Using an adjusted 'alpha' of 0.025 (0.05/2), a power of 80%, and a two-sided test, we find that 11 animals are needed per group when considering the difference of the means and a coefficient of variance of both 50%. We add an extra 7 animals to account for a possible loss of animals during the experiment. We believe that a loss of approximately 10%, due to systemic effects of infection, is a realistic estimation [2,3,4].

STUDY 3, the effect of controlled release of the inorganic nanoparticles (e.g. silver and zinc) (97 rats):

1) Implant with nanotubes (NT) (N=11 x 4 and 8 weeks)
2) Implant with NT + hydrogel (N=11 x 4 and 8 weeks)
3) Implant with NT + zinc in hydrogel (N=11 x 4 and 8 weeks)
4) Implant with NT + silver in hydrogel (N=11 x 4 and 8 weeks)

Groups 3 and 4 are compared to group 2 at the two different time points (no repeated measurements).
Using an adjusted 'alpha' of 0.025 (0.05/2), a power of 80%, and a two-sided test, we find that 11 animals are needed per group when considering the difference of the means and a coefficient of variance of both 50%. We add an extra 9 animals to account for a possible loss of animals during the experiment. We believe that a loss of approximately 10%, due to systemic effects of infection, is a realistic estimation [2,3,4].

STUDY 4, the effect of layer-by-layer release of antimicrobials (e.g. gentamicin) and growth factors (e.g. BMP-2) (100 rats).
1) Untreated implant (N=13)
2) Implant + growth factor (e.g. BMP-2) low concentration (N=13)
3) Implant + growth factor (e.g. BMP-2) high concentration (N=13)
4) Implant + antimicrobial (e.g. gentamicin) low concentration (N=13)
5) Implant + antimicrobial (e.g. gentamicin) high concentration (N=13)
6) Implant + growth factor (e.g. BMP-2)/antibiotic (e.g. gentamicin) low concentration (N=13)

7) Implant + growth factor (e.g. BMP-2) /antibiotic (e.g. gentamicin) high concentration (N=13)

The best layer-by-layer designs will be compared to the effect of the individual components (group 6 vs. group 2 and 4; group 7 vs. group 3 and 5). Thus, 4 comparisons are made. Using an adjusted 'alpha' of 0.0125 (0.05/4), a power of 80%, and a two-sided test, we find that 13 animals are needed per group when considering the difference of the means and a coefficient of variance of both 50%. We add an extra 9 animals to account for a possible loss of animals during the experiment. We believe that a loss of approximately 10%, due to systemic effects of infection, is a realistic estimation [2,3,4].

STUDY 5, the effect of the optimal bacterial-killing and bone promoting coatings (176 rabbits)

1) Untreated implant (N=12 x two time points)
2) Implant with nanotubes (N=12 x two time points)
3) Implant with Zinc or Silver (N=12 x two time points)
4) Implant with Zinc or Silver in hydrogel (N=12 x two time points)
5) Implant with growth factor (e.g. bone morphogenetic protein 2) (N=12 x two time points)
6) Implant with antimicrobial (e.g. gentamicin) (N=12 x two time points)
7) Implant with growth factor (e.g. bone morphogenetic protein 2)/antimicrobial (e.g. gentamicin) in layer-by-layer design (N=12 x two time points).

At each time point, group 4 is compared to group 3, and group 7 is compared to group 5 and 6 (no repeated measures). Thus, 3 comparisons are made. Using an adjusted 'alpha' of 0.0167 (0.05/3), a power of 80%, and a two-sided test, we find that 12 animals are needed per group when considering the difference of the means and a coefficient of variance of both 50%. We add an extra 8 animals to account for a possible loss of animals during the experiment. Based on our previous experience with this model (DEC 2013.III.11.083), we believe that a loss of approximately 5% of the rabbits, due to the systemic effects of infection, is a realistic estimation.

In total, we will therefore need 294 rats and 176 rabbits.

REFERENCES

[1] Reizner, JG Hunter, NT O'Malley, RD Southgate, EM Schwarz, SL Kates. A systematic review of animal models for Staphylococcus aureus osteomyelitis. Eur Cell Mater. 2014 Mar 25;27:196-212.
[2] Lucke et al. A new model of implant-related osteomyelitis in rats. J Biomed Mater Res B Appl Biomater. 2003 Oct 15;67(1):593-602.
[3] Poeppl et al. Daptomycin, Fosfomycin, or Both for Treatment of Methicillin-Resistant Staphylococcus aureus Osteomyelitis in an Experimental Rat Model. Antimicrob Agents Chemother. 2011 Nov; 55(11): 4999–5003.
[4] Poeppl et al. Efficacy of Fosfomycin Compared to Vancomycin in Treatment of Implant-Associated Chronic Methicillin-Resistant Staphylococcus aureus Osteomyelitis in Rats. Antimicrob Agents Chemother. 2014 Sep; 58(9): 5111–5116.

B. De dieren

Benoem de diersoorten, herkomst, geschatte aantallen en levenstadia. Onderbouw deze keuzes.

Rats:
Male Sprague Dawley, age 12-16 weeks (Charles River) . We expect to use 294 rats (see Statistical Methods).

The rat is a good animal model for our screening studies due to their small size, low cost, and established models of developing osteomyelitis described in literature [1,2]. Sprague Dawley rats have been used most often. By selecting the Sprague Dawley rats, we can base our bacteria inoculation dose on the literature.
Male rats are preferred of their larger size, which allows for larger K-wires to be implanted into the tibia, offering a larger surface area of the coating. The age of the rats is subsequently selected to ensure enough space in the tibial cavity for the placement of the k-wires (0.8 Ø x 25). Furthermore, the estrogen levels in female rats can fluctuate due to stress and age [3], and can affect bone regeneration [3,4]. Futhermore, it has been shown that male an female rats show a difference in their bone metabolism [5, 6,7]. If male and female rats are studied in a mixed population, this will cause a strong increase in the observed variance. We therefore prefer to use male rats only. The male rats can be housed together without problems.

Rabbits:
Female New Zealand White, age 12-16 weeks (Charles River). We expect to use 176 rabbits (see Statistical Methods).

Our group has previously performed dose-response studies with S. aureus in this adolescent rabbit model. Female rabbits can be used, which allows for grouped housing. Although, based on literature, both male and female rabbits can be used for this kind of research, we would like to use a single sex of rabbits. First, this minimizes the variance in the experiments, allowing the use of less animals. Second, we can compare our data to the results we obtained previously with this model. The selected age of the rabbits allows for the implantation of more clinically relevant-size implants (4 mm Ø x 25 mm). Furthermore, the adolescent age of the rabbits allows for in vivo scanning as this is problematic for larger animals, To our best knowledge, current literature does not suggest that adolescent rabbits may be more suitable as an osteomyelitis model than mature rabbits, or vice versa.

REFERENCES

1. W. Reizner, J.G. Hunter, N.T. O'Malley, R.D. Southgate, E.M. Schwarz, and S.L. Kates. A systematic review of animal models for Staphylococcus aureus osteomyelitis. Eur Cell Mater. 2014; 27: 196–212.
2. Patel M, Yuri Rojavin, Amir A. Jamali, Samantha J. Wasielewski, and Christopher J. Salgado. Animal Models for the Study of Osteomyelitis. Semin Plast Surg. 2009 May; 23(2): 148–154.
3. Hong L, Sultana H, Paulius K, Zhang G. Steroid regulation of proliferation and Biochem Mol Biol. 2009 Apr;114(3-5):180-5. PubMed PMID: 19429449. Pubmed Central PMCID: 2682591.
4. Calis M, Demirtas TT, Atilla P, Tatar I, Ersoy O, Irmak G, et al. Estrogen as a novel agent for induction of adipose-derived mesenchymal stem cells for osteogenic differentiation: in vivo bone tissue-engineering study. Plastic and reconstructive surgery. 2014 Apr;133(4):499e-510e. PubMed PMID: 24675202.
5. Sample SJ, Racette MA, Hao Z, Thomas CF, Behan M, Muir P. Functional adaptation in female rats: the role of estrogen signaling. PloS one. 2012;7(9):e43215. PubMed PMID: 22984413. Pubmed Central PMCID: 3439425.
6. Strube P, Mehta M, Baerenwaldt A, Trippens J, Wilson CJ, Ode A, Perka C, Duda GN, Kasper G. Sex-specific compromised bone healing in female rats might be associated with a decrease in mesenchymal stem cell quantity. Bone. 2009 DecL45(6):1065-72

C. Hergebruik

Is er hergebruik van dieren?

☒ Nee, ga door met vraag D.

☐ Ja > Geef aan op basis van welke overwegingen hergebruik in dit geval acceptabel wordt geacht.

Is er in het voorgaande of in het geplande gebruik sprake van (of een risico van) ernstig ongerief?

☒ Nee

☐ Ja > Geef aan op basis van welke overwegingen hergebruik in dit geval acceptabel wordt geacht.

D. Vervanging, vermindering en verfijning

Laat zien hoe de toepassing van methoden voor vervanging, vermindering en verfijning zijn meegewogen bij het bepalen van de experimentele strategie, de keuze van de dieren en de opzet van de dierproef en welk keuzes daarbij zijn gemaakt.

Replacement:
The effects of the implant coatings are first studied in vitro. For this purpose, we use a cytotoxicity assay. Also, bacterial proliferation and biofilm formation can be assessed in vitro. The best-performing conditions will subsequently be tested in vivo, as indirect effects by immune cells is hard to mimick in an in vitro model.

Reduction:
For each experiment, a power analysis will be performed to determine the minumum number of animals required. The inter-animal variation will be minimized so that smaller group sizes are needed to reach statistical significance. Rats with the same sex and age will be used.
By performing in vivo scanning in the animals, the effects of the infection on bone changes can be assessed at different time points without the need of different animals for different time points (not possible if bacterial culture has to be performed at different time points).

Refinement:
- Animals will be given 1 week to acclimatize to their new environment
- Animals have cage enrichment
- Animals are weighed weekly to monitor welfare
- Animals receive appropriate analgetics and anaesthetics (in consultation with the designated veterinarian)
- The animals are housed together as long as no signs of agression towards each other exist
- Daily scoring of the animals will be performed after the surgeries. Pain medication will be continued if necessary. For rabbits, this is a standard procedure. For rats, the extra handling needed for scoring will be requested in the working protocol
- In vivo scanning will be performed under adequate anaesthetics (in consultation with the designated veterinarian)

- During and after surgery, the animals will be placed on heat blankets
- Eye ointment will be used to prevent dry eyes of the animals during surgery
- Skin wounds will be sutured intracutaneously to prevent opening

Geef aan welke maatregelen zijn genomen om de kans op pijn, lijden of angst bij de dieren en de kans op nadelige milieueffecten tot een minimum te beperken.

The animals will have at least 1 week to acclimatize to the new environment in the GDL. The animals will be housed per two as much as possible. The respiration of the rats will be monitored by the researcher during surgery. The rabbits will be monitored for respiratory and heart function by the designated biotechnician. The animals will be placed on heat blankets after surgery and returned to routine housing after recovery of anethesia. Unrestricted weight bearing and activity will be allowed after surgery. Food is given ad libitum. The animals will be scored weekly by the animal caretakers or the researcher (in consultation with each other). After surgeries, this frequency will be increased. In the case of unexpected complications, the animal caretakers and the designated veterinarian are consulted.

Herhaling en duplicering

E. Herhaling

Geef aan hoe is nagegaan of deze dierproeven niet al eerder zijn uitgevoerd. Indien van toepassing geef aan waarom duplicatie noodzakelijk is.

These coating methods have been newly developed by our group. With the current literature, we are unable to predict the performance of our coating methods in an in vivo model.

Huisvesting en verzorging

F. Huisvesting en verzorging

Worden de dieren anders dan volgens de eisen in bijlage III van de richtlijn 2010/63/EU gehuisvest en/of verzorgd?

☒ Nee

☐ Ja > Geef, indien dit kan resulteren in nadelige effecten op het dierenwelzijn, aan op welke wijze de dieren worden gehuisvest en verzorgd en motiveer de keuze om af te wijken van de eisen in bovengenoemde bijlage III.

G. Plaats waar de dieren worden gehuisvest

Worden de dierproeven geheel of gedeeltelijk uitgevoerd bij een inrichting die niet onder de rechtstreekse verantwoordelijkheid van een instellingsvergunninghouder Wod valt?

☒ Nee > Ga verder met vraag H.

☐ Ja > Geef aan wat voor bedrijf of instelling dit betreft.

Waarom is hiervoor gekozen en hoe wordt een adequate huisvesting, verzorging en behandeling van de dieren gewaarborgd?

Ongeriefinschatting / humane eindpunten

H. Pijn en pijnbestrijding

Valt te voorzien dat er pijn kan optreden bij de dieren?

☐ Nee > Ga verder met vraag I.

☒ Ja > Worden in dat geval verdoving, pijnstilling en/of andere pijnverlichtingsmethoden toegepast?

☐ Nee > Motiveer dan waarom geen pijnverlichtingsmethoden worden toegepast.

☒ Ja

I. Overige aantasting van het welzijn en maatregelen

Welke eventuele andere vormen van welzijnsaantasting worden voorzien?

Moderate stress (cumulative) is estimated based on the following procedures:
- Moderate stress due to the surgery. Animals will experience pain in the treated joint. The animals might avoid loading of the limb after surgery. Bacterial infection casuses local and systemic signs of inflammation. Animals will have reduced food intake and experience weight loss after surgery. In previous experiments, the animals were loading the affected limb a few days after surgery.
- Analgetics: mild stress. Animals experience lameness and reduced food intake due to the injection analgetics.
- Scoring of the animals after surgery: mild stress. Extra handling of the animals for weighing and scoring
- In vivo scanning (max. 3 times depending on the duration of the experiment): mild to moderate stress. Due to handling and anesthetics.
- Euthanasia: mild stress due to handling
- Blood harvesting (rabbits, max. 5 times depending on the duration of the experiment): moderate stress due to local ear irritation and stress during handling (procedure is mild, but is considered as moderate because it is repeated)
- Fluorochrome injections (max. 3 times depending on the duration of the experiment): moderate stress due to local skin irritation and stress during han dling (procedure is mild, but is considered as moderate because it is repeated)

Geef aan wat de mogelijke oorzaken hiervan zijn.

All described measurements are needed to create the least harm for the animals and most secure outcomes for this project.

Beschrijf welke maatregelen worden genomen om deze schadelijke effecten te voorkomen of waar mogelijk te minimaliseren.

1. At least 1 week to acclimatize to the new environment
2. Adequate use of analgetics (in consultation with the designated veterinarian)
3. Adequate use of anesthetics (in consultation with the designated veterinarian)
4. Observation of vital signs during surgery.

rats: depth or narcosis, respiration, tail pinch test
rabbits: monitoring of respiratory and heart function by the biotechnical. The depth or narcosis (twitching/movement) can additionally be observed by the researcher performing the surgery.

5. Scoring of the animals daily after surgery
6. Euthanasia according to one of the methods listed in Appendix IV of directive 2010/63/EU.
7. Pain medication, starting before surgery, and continued for two days standard. If there is any indication, pain medication will be continued beyond this period.

J. Humane eindpunten

Valt te voorzien dat zich bij deze dierproef omstandigheden voordoen waarbij het toepassen van humane eindpunten geïndiceerd is om verder lijden van de dieren te voorkomen?

☐ Nee > Ga verder met vraag K.

☒ Ja > Geef aan welke criteria hierbij worden gehanteerd.

- The rats will be euthanized in case one or more of the following humane endpoints are observed:
  - Weight loss (criteria determined in consultation with the veterinarion)
  - Severe lethargy
  - Severe tachycardia
  - Severe tremor
- Possible other signs of sepsis
- Loss of the animals' ability to walk and feed themselves
- Unexpected wound complications (such as non-closing and severe pus formation)

- The rabbits will be euthanized in case one or more of the following humane endpoints are observed:
  -Weight loss (criteria determined in consultation with the designated veterinarian)
  - Severe lethargy
  - Severe tachycardia
  -Severe tremor
  - Possible other signs of sepsis
  - Loss of the animals' ability fo walk and feed themselves
  -Joint infection with severe pus formation.

Welk percentage van de dieren loopt kans deze criteria te halen?

Rats: unlikely, max. 10%. The chance is higher in the pilot study, max. 20%.
Rabbits: very unlikely, max. 5%.

K. Classificatie van ongerief

Geef aan hoe in het licht van alle hierboven beschreven negatieve effecten het cumulatief ongerief wordt geclassificeerd in termen van '*terminaal*', *'licht'*, '*matig'* of *'ernstig'* ongerief.

The procedure is classified as moderate.

Einde experiment

L. Wijze van doden

Worden de dieren als onderdeel van het experiment of na afloop van het experiment gedood?

☐ Nee > Ga verder met de ondertekening.

☒ Ja > Geef aan waarom het doden van dieren als eindpunt essentieel is voor deze proef.

The animals need to be euthanized to harvest the bone and implant for bacterial culture.

Wordt er een methode(n) van doden uit bijlage IV van richtlijn 2010/63/EU toegepast?

☐ Nee > Beschrijf de euthanasiemethode en onderbouw de keuze hiervoor.

☒ Ja

Bijlage
Beschrijving dierproeven

- Deze bijlage voegt u bij uw projectvoorstel dierproeven.
- Per type dierproef moet u deze bijlage invullen en toevoegen.
- Meer informatie vindt u op de website www.zbo-ccd.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Vul uw deelnemernummer van de NVWA in.	11500
1.2	Vul de naam van de instelling of organisatie in.	UMC Utrecht
1.3	Vul het volgnummer en het type dierproef in. *Gebruik de volgnummers van vraag 3.4.4 van het format Projectvoorstel.*	Volgnummer: 2 Type dierproef: Spinal fusion in rats and rabbits in combination with ectopic implants

2 Beschrijving dierproeven

A. Experimentele aanpak en primaire uitkomstparameters

Beschrijf de keuze van de experimentele aanpak en de primaire uitkomstparameters.

We have previously identified inflammation-associated cytokines and bacterial-derived factors that could serve as bone-stimulating coatings on ceramics or metals intended for various orthopedic applications [1,2,3]. We more recently also showed that the use of components derived from bacteria is potentially a safe and effective way to stimulate bone formation (unpublished). These biological factors enhance bone formation by causing a beneficial immune response.

Currently, the transplantation of patient's own bone is used to stimulate bone formation in patient's when needed, including non-healing fractures, filling of large bone defects, or the re-alignment of the skeleton [4]. There are however a lot of complications associated with the bone harvesting procedure and there

is only a limited amount of bone available for the surgeon. The coating of materials with the aforementioned biological factors could be an alternative way to stimulate de novo bone formation or to improve the bone integration of implants. The fusion of vertebrae- e.g. during abnormal curvature of the spine or due to chronic back pain- is the most important application for which bone grafts are needed [6].

We aim to test the efficacy of our coatings on the bone formation in a spinal fusion model in rats and rabbits [7, 8]. As spinal fusion is the most important application for which bone grafts are used, we would like to test the efficacy of our coatings in the same setting. A calcium phosphate ceramic will be used (e.g. biphasic calcium phosphate BCP or tricalcium phosphate TCP). These materials are promosing for use in spinal fusion because of their ability to conduct bone formation along their surface. If bone-stimulating coatings are identified in the spinal fusion model, these can directly be tested for their ability to improve bone integration in the implant infection models.

The spinal fusion procedure is relatively simple to perform in these animals, in contrast to the spinal fusion technique in humans or larger animal models, where instrumentation is required. The rat spinal fusion model allows for better study of the underlying processes as there are more research tools available for rodents compared to rabbits and larger animals. The rabbit model on the other hand allows for better translation to the clinic, as larger constructs can be tested and the immune system is more comparable to humans. When using inflammation-associated cytokines or components derived from bacteria, the immune response is an important outcome parameter. We believe that this is more representative of the human situation in rabbits than it is in rats. Furthermore, in rabbits, a comparison can be made between our coating method and the current gold standard in the clinic (e.g. autologous bone harvested from the hip bone). This is difficult to perform in rats due to their smaller size.

The implantation of ectopic constructs in the same animals allows for additional studies to be performed in parallel to the functional spinal fusion test in the orthotopic location.
First, in an ectopic location, bone formation is only the result of osteoinduction (i.e. differentiation of stem cells into bone cells), whereas osteoconduction (i.e. bone outgrowth from existing bone) also plays a role in the orthotopic location. By combining the ectopic and orthotopic sites, the potential of the inflammatory compounds for osteoinduction and osteocondution can be both studied. This can elucidate the potential use of the compounds in different applications and locations.
Second, the optimal dose of in vitro-indentified inflammatory compounds can be studied in an ectopic location before testing them in an orthotopic location. Because there are more implantations possible in the ectopic site compared to the orthotopic site, we can also try to identify new bone-stimulating inflammatory compounds in the ectopic location which can be tested in follow-up studies. For the first in vivo studies, the optimal dose of interesting inflammatory compounds have already been determined ectopically in a previous study and can be directly tested in an orthotopic location.
By combining the ectopic and orthotopic locations, the animals are used in an optimal way and multiple research questions can be answered within the same study.

Several outcome parameters will be studied related to new bone formation and inflammation. In vivo micro-CT scans are performed under general anesthesia to quantify new bone formation at different time points. Fluorochrome markers are injected at different time points to trace the onset and progression of bone formation. After euthanization of the animals, local inflammation markers are measured on tissue samples. For the ectopic implants, quantification of the bone staining in histological samples is performed. For the spinal fusion implants, a combination of different methods is used to assess the degree of fusion between the vertebrae, including manual palpation, ex vivo micro-CT imaging, and quantification of the bone tissue in histological samples. In the rabbit studies, the systemic effects of the coatings will also be assessed by measuring systemic markers of inflammation on blood samples. Local inflammation markers are also measured on the spinal fusion masses.

References:

1. M Croes, F. Cumhur Oner, Moyo C. Kruyt, Taco J. Blokhuis, Okan Bastian, Wouter J.A. Dhert, Jacqueline Alblas. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. Plos one 2015.
2. M Croes, W Boot, B Pouran, MC Kruyt, D Gawlitta, Y van der Helm, C Vogely, H Weinans, J Alblas, WJA Dhert, FC Oner. Bacterial infection increases bone volume in a rabbit osteomyelitis model. 2015. Submitted
3. M Croes, W Boot, MC Kruyt, WJA Dhert, B Pouran, FC Oner, J Alblas. Bacteria-associated inflammation induces bone formation in a pre-clinical rabbit model. In preparation
4. PV Giannoudis, H Dinopoulos, E Tsiridis : Bone substitutes: An update. Injury 2005.
5. BM Desai. Osteobiologics. Am J Orthop 2007
6. WG De Long, TA Einhorn, K Koval et al. Current concepts review: Bone grafts and bone graft substitutes in orthopaedic trauma surgery. J Bone Joint Surg AM 2007
7. SD Boden, GJ Martin, M Morone, JL Ugbo, L Titus, WC Hutton. The use of coralline hydroxyapatite with bone marrow, autogenous bone graft, or osteoinductive bone protein extract for posterolateral lumbar spine fusion. Spine. 1999;24:320–327
8. Drespe et al. Animal models for spinal fusion. Spine J. 2005.

Beschrijf de beoogde behandeling van de dieren (inclusief de aard, de frequentie en de duur van de behandelingen waaraan de dieren worden blootgesteld) en onderbouw de gekozen aanpak.

Surgical procedures

Ectopic implantations: Using a single skin midline incision in the dorsum, PMMA (poly methyl methacrylate) discs are implanted intramuscularly and subcutaneously. We have previously used up to 18 samples in rabbits and up to 14 samples in rats, leaving enough space beteen the samples to minimize cross-over effects between samples. After 6 weeks, all PMMA discs are removed from their surrounding membranes ('biomembranes'), and replaced scaffolds (calcium phosphate ceramics or porous metal implant) coated with the factors of interest. These 'biomembranes' act as a physical barrier, preventing initial leakage of growth factors or inflammation-associated factors to the surrounding tissues [1]. As a results, one creates a more localized inflammatory response and limits the cross-over effects to neighbouring implants. The first procedure (implantation of the spacer) is expected to take approximately 1 hour, while the second procedure (implantation of the loaded material in the 'biomembrane') is thought to take approximately 1.5 hours. The implantation of the loaded constructs is always performed in a randomized way.

The membranes act as a physical barrier, preventing initial leakage of the delivered components which could effect neighbouring scaffolds. The skin wounds are closed with resorbable sutures. During the experiment, fluorochrome markers are injected subcutaneously at max. three time points to determine the progression of bone formation by fluorescence microscopy. In vivo micro-CT scans are performed under general anesthesia to quantify the progress of bone formation (max. 3 times). The animals are euthanized 8-12 weeks after the second surgery to retrieve the implants for analyses by histology. This exact end point (8-12 weeks) is chosen for each experiment separately, but is determined by the speed of bone formation which is expected (i.e. if bone stimulating growth factors such as BMPs are used), and will be minimized.

This model has been used by our group in both species successfully without loss of animals. In rabbits, blood is harvested from the ear vein at max. 5 time points to measure systemic markers of inflammation.

Spinal fusion: this procedure is combined with the second surgery for the ectopic implantations at week 6. The dorsal midline skin incision is followed by two paramedian fascial incisions between the multifidus and longissimus muscles until the transverse processes in the lumbar region are exposed. The exposed transverse processes are decorticated bilaterally with an electric burr. On both sides of the vertebra, the graft of interest is implanted next to the spine. This involves a calcium phosphate ceramic or a porous metal implant in the experimental group, with or without coating with the biological factor of interest. When a comparison is made with the current gold standard in the clinic (i.e. autologous bone), separate fascial incisions are used to harvest corticocancellous bone from the iliac crest bone to place between the transverse processes in the same way. The spinal fusion procedure can be performed at a single level or at two levels of the vertebrae [1,2, 3]. The skin wound is closed with resorbable sutures. The animals are euthanized (see ectopic implantations) for ex vivo micro-CT scanning and the fusion masses/ectopic implants are retrieved for histological analyses. Local inflammation markers are measured in the fusion masses.

Bone regeneration measurements

The primary outcome parameter is the amount of bone formation after 8-12 weeks. For the ectopic implants, quantification of the bone in histological samples is the best method. For the spinal implants, a combination of techniques is used to assess the degree of fusion between the vertebrae, including manual palpation, in vivo and ex vivo micro-CT, and quantification of the bone tissue in histological samples. Animals will receive fluorochrome markers subcutaneously to determine the onset and localization of new bone formation by fluorescence microscopy post-mortem.

Inflammation measurements

In the rabbits, blood will be harvested at max. 5 time points to measure systemic markers of inflammation. Local inflammation markers will be measured on tissue samples after euthanization.

Animal stress

The animals are estimated to experience mild stress (cumulative). The procedures are expected to take approximately 1 hour (first surgery) and 2 hours (second surgery).

Anaesthesia protocol

Rats: Start and maintained with isoflurane/oxygen.
Rabbits: a combination of injection analgetics/anesthetics is used. The protocols for adequete anaesthesia are developed together with the veterinarian.

Pain management

All animal receive injection analgetics. The protocols used for pain management will be developed together with the designated veterinarian. If any

unexpected signs on pain continue to exist in the first days after the operational procedure, as observed during daily scoring of the animals, the designated veterinarian will be consulated for the adequate pain management.

Antiseptic techniques

All operations will be performed aseptically conditions. The surgeon will wear scrubs, sterile gloves and sterile gown. The surgeon will wear a surgery mask and cap. Only autoclaved instruments will be used. After shaving, the skin will be disinfected with Providone Iodine. All materials introduced into the animals are autoclaved or filtered to ensure sterility.

Postoperative care

The animals will be placed on heat blankets post-operatively. The animals will be housed per two, unless there are signs of agression towards each other. The animals will be scored daily for one week after the operational procedure. The weight of the animals will be measured once a week. The animals will receive injection pain medication for two days at minimum, and longer if necessary.

Euthanasia protocols

Euthanasia is performed according to one of the methods listed in appendix IV of directive 2010/63/EU. The animals will be euthanized 8-12 weeks after the second surgery . This involves CO2 inhalation in rats, and an overdose of Pentobarbital i.v. in rabbits under the same general anaesthesia and pain medication as the surgery procedure.

References:

1. Viateau V, Guillemin G, Bousson V, Oudina K, Hannouche D, Sedel L, et al. Long-bone critical-size defects treated with tissue-engineered grafts: a study on sheep. J Orthop Res 2007;25: 741-9
2. SD Boden, GJ Martin, M Morone, JL Ugbo, L Titus, WC Hutton. The use of coralline hydroxyapatite with bone marrow, autogenous bone graft, or osteoinductive bone protein extract for posterolateral lumbar spine fusion. Spine. 1999;24:320–327
3. AM Riordan, R Rangarajan, JW Balts, WK Hsu, PA Anderson. Reliability of the Rabbit Postero-Lateral Spinal Fusion Model: A Meta-Analysis. JOR 2013
4. Drespe et al. Animal models for spinal fusion. Spine J. 2005.

Geef aan welke overwegingen en statistische methoden worden gebruikt om het aantal benodigde dieren tot een minimum te beperken.

The following section describes the minimal number of animals needed for each study. For the rabbit study, a 5% loss of animals is estimated. This percentage is based on literature. As this loss in the rabbits is mainly the result of the autologous bone harvesting procedure, it is not expected in the rat studies.

The outcome parameter on which the group size are based is the success of spinal fusion (yes or no) as determined by micro-CT. The difference of the means and the coefficient of variance of treatment with a biological coating in the spinal fusion model is unknown [1]. We can therefore only determine the minimal

group size based on a rough estimation of these parameters.

In a typical experimental design, 3 groups are used. We will perform a one way ANOVA's with a Bonferroni post-hoc correction. The alpha is adjusted according to two comparisons that are made (experimental group vs. positive control and negative control OR two experimental groups vs. positive control). For example:

1) Positive control group, autograft or bone morphogenetic protein (BMP)
2) Negative control group (e.g.calcium phosphate ceramic such as biphasic calcium phosphate BCP or tricalcium phosphate TCP)
3) Experimental group (e.g. calcium phosphate ceramic coated with proinflammatory cytokine or bacterial-derived factor)

Using an adjusted 'alpha' of 0.025 (0.05/2), a power of 80%, and a two-sided test, we find that 11 animals are needed per group when considering the difference of the means and a coefficient of variance of both 20%. We hereby consider a difference of the means of at least 20% to be of clinically relevance.

We expect to perfom the following studies in the upcoming 5 years:

STUDY 1, the effect of inflammation-associated cytokines on bone formation in rats (33 rats):
Group 1. Pos control, calcium phosphate with BMP, N=11
Group 2. Neg control, empty calcium phosphate (e.g. BCP or TCP), N=11
Group 3. Experimental group, calcium phosphate coated with proinflammatory cytokines, N=11

We have already determined the optimal concentration of a number inflammatory compounds in an ectopic location. These can be studied in the orthotopic location in study 1. Simultaneously, we can perform dose studies for bacterial components determined in vitro in the ectopic location in the same animals. These can then be tested in the orthotopic location in study 2.

STUDY 2, the effect of bacterial components on bone formation in rats (33 rats):
Group 1. pos. control, calcium phosphate with BMP, N=11
Group 2. Experimental group 1, calcium phosphate coated with bacterial component 1, N=11
Group 3. Experimental group 2, calcium phosphate coated with bacterial component 2, N=11

The optimal concentration of bacterial components are determine in the ectopic location in study 1. These can be studied in the orthotopic location in study 2. Simultaneously, we can perform dose studies for future experiments in the ectopic location in study 2.

STUDY 3, the effect of inflammation-associated cytokines on bone formation and systemic inflammation markers in rabbits (35 rabbits):
Group 1. Pos control, autologous bone, N=11
Group 2. Neg control, empty calcium phosphate, N=11
Group 3. Experimental group, calcium phosphate coated with inflammation-associated cytokines, N=11

For this rabbit study, we add an extra 2 animals to account for a possible loss of animals during the experiment. We believe that a loss of approximately 5%,

due to deep wound infections or autologous bone harvest-associated nerve damage and blood loss, is a realistic estimation [2-6].

We have already determined the optimal concentration of a number inflammatory compounds in an ectopic location. These can be studied in the orthotopic location in study 3. Simultaneously, we can perform dose studies for bacterial components determined in vitro in the ectopic location in the same animals. Subsequently, the optimal dose can be tested in the orthotopic location in study 4.

STUDY 4, the effect of bacterial components on bone formation and systemic inflammation markers in rabbits (35 rabbits) :

Group 1. Pos control, autologous bone, N=11
Group 2. Experimental group 1, calcium phosphate coated with bacterial component 1, N=11
Group 3. Experimental group 2, calcium phosphate coated with bacterial component 2, N=11

For this rabbit study, we add an extra 2 animals to account for a possible loss of animals during the experiment. We believe that a loss of approximately 5%, due to deep wound infections or autologous bone harvest-associated nerve damage and blood loss, is a realistic estimation [2-6].

The optimal concentration of bacterial components are determine in the ectopic location in study 3. These can be studied in the orthotopic location in study 4. Simultaneously, we can perform dose studies for future experiments in the ectopic location in study 4.

Thus, we estimate that in total 66 rats and 70 rabbits are needed.

REFERENCES

1. AM Riordan, R Rangarajan, JW Balts, WK Hsu, PA Anderson. Reliability of the Rabbit Postero-Lateral Spinal Fusion Model: A Meta-Analysis. JOR 2013
2. Bransford et al. Effect of zoledronic acid in an L6–L7 rabbit spine fusion model. Eur Spine J. 2007 Apr; 16(4): 557–562.
3. Cinotti et al. Experimental posterolateral spinal fusion with porous ceramics and mesenchymal stem cells. J Bone Joint Surg Br. 2004 Jan;86(1):135-42.
4. Lee et al. The effect of acute smoking on spinal fusion: an experimental study among rabbits. J Trauma. 2005 Aug;59(2):402-8.
5. Lehman et al. The effect of alendronate sodium on spinal fusion: a rabbit model. Spine J. 2004 Jan-Feb;4(1):36-43
6. Long et al. The effect of cyclooxygenase-2 inhibitors on spinal fusion. J Bone Joint Surg Am. 2002 Oct;84-A(10):1763-8.

B. De dieren

Benoem de diersoorten, herkomst, geschatte aantallen en levenstadia. Onderbouw deze keuzes.

We would like to use both rats and rabbits for our experiments. There are more research tools (e.g. antibodies) available for the rat species, allowing for more mechanistic studies. The rabbits on the other hand allow for more translational research: safety studies can be performed due to their comparable immune system to humans, a comparison can be made between our coating and the golden standard (i.e. autologous bone grafting) and larger constructs can be tested in rabbits

Rats: male Fischer 344, 10-16 weeks (Charles River). We estimate to use 66 rats (see Statistical Methods).

The Fischer 344 rat has been used as a spinal fusion model before [1]. Our ectopic rat model also makes use of this male rat strain, wich allows for comparison of the data when using calcium phosphates or BMP-2. Furthermore, in this model we can make a good prediction of the amount of bone formation in the control group, which allows us to select the appropriate timepoints for analyses. The estrogen levels in female rats can fluctuate due to stress and age [2], and can affect bone regeneration [3,4]. Futhermore, it has been shown that male an female rats show a difference in their bone metabolism [5,6]. If male and female rats are studied in a mixed population, this will cause a strong increase in the observed variance. We therefore prefer to use male rats only. As an advantage, we have experienced that the male Fischer 344 rats can be housed together without problems [5,6]

Rabbits: adult female New Zealand White, 20-24 weeks (Charles River). We estimate to use 70 rabbits (see Statistical Methods).
The rabbit spinal fusion model has been used frequently, as the fusion rate with autologous bone is similar to humans. A number of characteristics of the rabbits (age, weight, gender, species) have been tested on the rate of fusion in a meta-analysis study [7]. This study shows that the use of female New Zealand white rabbits is very suitable. For our research this is beneficial, as we previously studied the biological coating components in female New Zealand white rabbits too. However, we wish to keep the sex within our spinal fusion model fixed, due to the 20% difference in the fusion rate between male and female rabbits [7]. The increase in variance when using a mixed-gender population would results in a need for a larger sample size to demonstrate a statistically significant difference. Individual housing of male rabbits is associated with a decreased general well-being, therefore the use of females may be preferable. There is no evidence that fluctuating estrogen levels in female rabbits can affect the bone formation process [8]. In addition, the rabbit tibia model also makes use of female rabbits, therefore allowing better translation to the spinal fusion model. This meta-analysis furthermore shows that the animals should be at least 3 kg in weight, while there seems to be less of an effect for the age of the animals. Our choice of the age/weight allows for a sufficient number of ectopic implants in the dorsal region, with enough space between them to minimal cross-over effects between the samples.

Adult rabbits are used as this seems to be beneficial for the success of fusion. Furthermore, almost all studies described in literature make use of adult rabbits [7].

Generally speaking, the rabbit model is used in a final step before an intrumented spinal fusion model (e.g. sheep or goats). As such, larger constructs can be used and the rabbit immune system allows for prediction of dosis effects considering that its immune system is more comparable to humans than rodents [9, 10]. This is an important aspect to consider, as some of the biological coatings induce bone formation through an immune response.

References:

1. Lopez et al. Acceleration of Spinal Fusion Using Syngeneic and Allogeneic Adult Adipose Derived Stem Cells in a Rat Model. J Orthop Res 2009.
2. Arakawa K, Arakawa H, Hueston CM, Deak T. Effects of the estrous cycle and ovarian hormones on central expression of interleukin-1 evoked by stress in female rats. Neuroendocrinology. 2014;100(2-3):162-77. PubMed PMID: 25300872.
3. Hong L, Sultana H, Paulius K, Zhang G. Steroid regulation of proliferation and Biochem Mol Biol. 2009 Apr;114(3-5):180-5. PubMed PMID: 19429449. Pubmed Central PMCID: 2682591.
4. Calis M, Demirtas TT, Atilla P, Tatar I, Ersoy O, Irmak G, et al. Estrogen as a novel agent for induction of adipose-derived mesenchymal stem cells for osteogenic differentiation: in vivo bone tissue-engineering study. Plastic and reconstructive surgery. 2014 Apr;133(4):499e-510e. PubMed PMID: 24675202.
5. Sample SJ, Racette MA, Hao Z, Thomas CF, Behan M, Muir P. Functional adaptation in female rats: the role of estrogen signaling. PloS one. 2012;7(9):e43215. PubMed PMID: 22984413. Pubmed Central PMCID: 3439425.
6. Strube P, Mehta M, Baerenwaldt A, Trippens J, Wilson CJ, Ode A, Perka C, Duda GN, Kasper G. Sex-specific compromised bone healing in female rats might

be associated with a decrease in mesenchymal stem cell quantity. Bone. 2009 DecL45(6):1065-72
7. AM Riordan, R Rangarajan, JW Balts, WK Hsu, PA Anderson. Reliability of the Rabbit Postero-Lateral Spinal Fusion Model: A Meta-Analysis. JOR 2013
8. Mapara M, Thomas BS, Bhat KM/ Rabbit as an animal model for experimental research. Dent Res J (Isfahan). 2012 Jan-Mar; 9(1): 111–118
9.Copeland S, Warren HS, Lowry, SF, Calvano SE, Remick D. Acute Inflammatory Response to Endotoxin in Mice and Humans. Clin Diagn Lab Immunol. 2005 Jan; 12(1): 60–67
10. Jacquier V, Estellé, Schmaltz-Panneau B et al. Genome-wide immunity studies in the rabbit: transcriptome variations in peripheral blood mononuclear cells after in vitro stimulation by LPS or PMA-Ionomycin. BMC Genomics 2015, 16:26

C. Hergebruik

Is er hergebruik van dieren?

☒ Nee, ga door met vraag D.

☐ Ja > Geef aan op basis van welke overwegingen hergebruik in dit geval acceptabel wordt geacht.

Is er in het voorgaande of in het geplande gebruik sprake van (of een risico van) ernstig ongerief?

☒ Nee

☐ Ja > Geef aan op basis van welke overwegingen hergebruik in dit geval acceptabel wordt geacht.

D. Vervanging, vermindering en verfijning

Laat zien hoe de toepassing van methoden voor vervanging, vermindering en verfijning zijn meegewogen bij het bepalen van de experimentele strategie, de keuze van de dieren en de opzet van de dierproef en welk keuzes daarbij zijn gemaakt.

Replacement
The biological coatings we would like to test are thought to stimulate bone formation due to their immunogenic properties.
The complexity of the bone environment and the interaction between immune and bone cells are difficult to mimick in vitro. Although we and others try to predict their effects on bone cells in vitro [1], their actual effects on bone tissue in vivo effects seem to be very different [3,4]. However, in vitro studies are still performed where possible, for instance to determine the optimal coating methods for the in vivo studies. For this purpose, the differentiation of stem cells into bone cells can be tested on the different coated constructs in vitro.

Reduction

Although the spinal fusion model requires relatively large group sizes to demonstrate statistically significant differences, there are a number of ways by which the number of rabbits can be reduced.
First, ectopic implantations can be performed in parallel so that multiple research questions can be answered within one experiment. The ectopic screening model allows for a large number of implants to be studied. We have previously used up to 18 samples in rabbits. Furthermore, the subcutaneous and

intramuscular locations can be easily studied in parallel.
Second, mechanistic studies are first performed in a screening setting ectopically. As the spinal fusion requires large group sizes, only the optimal conditions will be tested here. As such, 2-3 groups in total are sufficient.
Third, spinal fusion at two vertebral levels means that 2 treatments can be compared to the control in the same study.

Refinement

- Skin wounds will be sutured intracutaneously, using resorbable sutures
- Animals are housed together as much as possible
- Food is given ad libitum (pellets and hay)
- Animals are weighed weekly to monitor health
- Animals will be given 1 week to acclimatize to their new environment
- Eye ointment will be used to prevent dry eyes during surgery
- During and after surgery the animals will be placed on heat blankets
- Animals will receive adequate anaesthetics to prevent harm during surgery
- Analgesia medication will be administered untill 2 days after surgery to prevent harm post-operatively
- Daily scoring of the animals after the operations. Pain medication will be continued if necessary. For rabbits, this is a standard procedure after the OR

References:

1. Mo et al. Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors. BMC Cell biology. 2008.
2. Croes et al. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. Plos One. 2015.
3. Croes et al. Local inflammation and bone formation in a new translational animal model. In preparation.
4. Croes et al. Bacterial infection increases bone volume in a rabbit osteomyelitis model. Submitted.

Geef aan welke maatregelen zijn genomen om de kans op pijn, lijden of angst bij de dieren en de kans op nadelige milieueffecten tot een minimum te beperken.

The animals will have at least 1 week to acclimatize to the new environment before surgery. Animals will be given food and water ad libitum. The rabbits will be monitored for the respiratory and heart function by the designated biotechnician. The animals will be placed on heat blankets postoperatively. The animals will be returned to routine housing after they have recovered from anesthesia. For specific information see 'refinement'.

The animals will be housed conform the standards of the GDL Utrecht. They will be housed in groups of two as much as possible. This has been done for female New Zealand White rabbits and male Fischer rats in the past, usually without problems. In cases when animals show signs of agression towards each other, the animals will be housed individually to prevent harm.
The animals are weighed weekly by the animal care takers or researcher and monitored weekly (written and signed off in the working plan). This observation frequency is higher (once daily) after the surgeries. When weight loss is suspected later in the experiment, the animals will be monitored for the humane

endpoints and weighed daily.

Herhaling en duplicering

E. Herhaling

Geef aan hoe is nagegaan of deze dierproeven niet al eerder zijn uitgevoerd. Indien van toepassing geef aan waarom duplicatie noodzakelijk is.

n.v.t.

Huisvesting en verzorging

F. Huisvesting en verzorging

Worden de dieren anders dan volgens de eisen in bijlage III van de richtlijn 2010/63/EU gehuisvest en/of verzorgd?

☒ Nee

☐ Ja > Geef, indien dit kan resulteren in nadelige effecten op het dierenwelzijn, aan op welke wijze de dieren worden gehuisvest en verzorgd en motiveer de keuze om af te wijken van de eisen in bovengenoemde bijlage III.

G. Plaats waar de dieren worden gehuisvest

Worden de dierproeven geheel of gedeeltelijk uitgevoerd bij een inrichting die niet onder de rechtstreekse verantwoordelijkheid van een instellingsvergunninghouder Wod valt?

☒ Nee > Ga verder met vraag H.

☐ Ja > Geef aan wat voor bedrijf of instelling dit betreft.

Waarom is hiervoor gekozen en hoe wordt een adequate huisvesting, verzorging en behandeling van de dieren gewaarborgd?

Ongeriefinschatting / humane eindpunten

H. Pijn en pijnbestrijding

Valt te voorzien dat er pijn kan optreden bij de dieren?

☐ Nee > Ga verder met vraag I.

☒ Ja > Worden in dat geval verdoving, pijnstilling en/of andere pijnverlichtingsmethoden toegepast?

☐ Nee > Motiveer dan waarom geen pijnverlichtingsmethoden worden toegepast.

☒ Ja

I. Overige aantasting van het welzijn en maatregelen

Welke eventuele andere vormen van welzijnsaantasting worden voorzien?

Cumulative moderate (matig) stress is estimated based on the following procedures:

-Operations: moderate stress. Due to anesthetics and ectopic implantation of samples and implantation next to the spinal column. Animals may experience pain in the dorsum after the operation. When autologous bone is used, the animals may experience pain in the hip bone from bone harvesting. Animals will have reduced food intake and have weight loss due to the operations.
-Daily scoring of animals after the operation: mild stress due to handling.
-Injection of pain medication subcutaneously: mild stress due to handling and injection.
-Injection of fluorochrome markers subcutaneously (max. 3 times depending on duration of experiment): moderate stress caused by handling and local irritation of the skin (procedure causes mild stress, but considered as moderate as it is repeated).
-In vivo micro-CT scans (max.3 times dependening on duration of experiment): moderate moderate stress due to handling and anesthetic induction (procedure causes mild stress, but is considered as moderate as it is repeated)
-Blood harvest (rabbits, max. 5 times depending on studied factor and duration of experiment): moderate due to handling and ear irritation (procedure causes mild stress, but it is considered as moderate as it is repeated).
Euthanasia: mild stress due to handling.

Geef aan wat de mogelijke oorzaken hiervan zijn.

All described measurements are needed to create the least harm for the animals and most secure outcomes for this project.

Beschrijf welke maatregelen worden genomen om deze schadelijke effecten te voorkomen of waar mogelijk te minimaliseren.

1. At least 1 week to acclimatize to the new environment before surgery
2. Adequate use of injection anesthetics during the implantation procedure
3. The depth of narcosis (twitching/movement) can also be observed by the researcher performing the procedure. In the case of rabbits, the respiratory and heart function will be monitored by the biotechnician assisting with the anesthetics during the OR.
4. Adequate observation of vital signs of the animals post-operatively. Scoring of the animals daily after operations by the animal caretakers and the researcher.
5. For euthanasia, one of the methods listed in Appendix IV of directive 2010/63/EU is used
6. Pain medication, starting before the operation, and continued for two days as standard. If there is an indication, pain medication will be extended.

J. Humane eindpunten

Valt te voorzien dat zich bij deze dierproef omstandigheden voordoen waarbij het toepassen van humane eindpunten geïndiceerd is om verder lijden van de dieren te voorkomen?

☐ Nee > Ga verder met vraag K.

☒ Ja > Geef aan welke criteria hierbij worden gehanteerd.

- The animals will be euthanized in case one or more of the following humane endpoints are observed:
- Weight loss. When weight loss is suspected (reduced food intake), the animals will be weighed daily to monitor welfare
- Severe lethargy
- Severe tachycardia
- Severe tremor
- Loss of the animals' ability to walk and feed themselves
- Persistent infection of wounds.

-

Welk percentage van de dieren loopt kans deze criteria te halen?

Rats: not expected.
Rabbits: very unlikely, max. 5%.

K. Classificatie van ongerief

Geef aan hoe in het licht van alle hierboven beschreven negatieve effecten het cumulatief ongerief wordt geclassificeerd in termen van '*terminaal'*, *'licht'*, *'matig'* of *'ernstig'* ongerief.

The procedure is classified as moderate (matig).

Einde experiment

L. Wijze van doden

Worden de dieren als onderdeel van het experiment of na afloop van het experiment gedood?

☐ Nee > Ga verder met de ondertekening.

☒ Ja > Geef aan waarom het doden van dieren als eindpunt essentieel is voor deze proef.

The animals need to be euthanized to harvest the implants (ectopic model) for histology.

Wordt er een methode(n) van doden uit bijlage IV van richtlijn 2010/63/EU toegepast?

☐ Nee > Beschrijf de euthanasiemethode en onderbouw de keuze hiervoor.

☒ Ja

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

UMC Utrecht
[redacted]
Postbus 12007
3501 AA Utrecht

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD115002016445

Uw referentie

Bijlagen
1

Datum 7 april 2016
Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 23 februari 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Smart coatings for orthopedic implants" met aanvraagnummer AVD115002016445. Wij hebben uw aanvraag beoordeeld.

Op 23 maart 2016 hebben wij u een vraag gesteld over het aantal dieren dat u beschrijft in de bijlagen dierproeven. De aantallen waren niet consistent weergegeven. U heeft de bijlagen dierproeven aangepast en deze op 29 maart 2016 naar ons toegestuurd.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). U kunt met uw project "Smart coatings for orthopedic implants" starten. De vergunning wordt afgegeven van 7 april 2016 tot en met 1 januari 2021. De startdatum wijkt af van uw aanvraag omdat deze in het verleden ligt.
Aanvullend stelt de CCD twee algemene voorwaarden om te voldoen aan datgene wat voort komt uit artikel 10a. van de wet.
Overige wettelijke bepalingen blijven van kracht.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC Utrecht gevoegd. Dit advies is opgesteld op 18 februari 2016. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet.
Wij nemen het advies van de DEC over, inclusief de daaraan ten grondslag liggende motivering. In aanvulling hierop worden de twee algemene voorwaarden gesteld die aan meerjarige projecten worden verbonden.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze gegevens in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

De Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163

Bijlagen
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam: UMC Utrecht
Adres: postbus 12007
Postcode en woonplaats: 3501 AA Utrecht
Deelnemersnummer: 11500

deze projectvergunning voor het tijdvak 7 april 2016 tot en met 1 januari 2021, voor het project "Smart coatings for orthopedic implants" met aanvraagnummer AVD115002016445, volgens advies van Dierexperimentencommissie DEC Utrecht.
De functie van de verantwoordelijk onderzoeker is PhD Onderzoeker. Voor de uitvoering van het project is Onderzoekster [redacted] verantwoordelijk:

De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 23 februari 2016
2. de bij het aanvraagformulier behorende bijlagen:
   a. Projectvoorstel, zoals ontvangen bij digitale indiening op 29 maart 2016;
   b. Niet-technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 23 februari 2016;
   c. Advies van Dierexperimentencommissie dd 18 februari 2016, ontvangen op 23 februari 2016
   d. De aanvullingen op uw aanvraag zoals ontvangen op 29 maart 2016.

Dierproeven

Naam dierproef	Diersoort	Aantal dieren	Ernst
Implant infection model in rats and rabbits	Ratten (Rattus norvegicus) / Spraque Dawley	294	Matig
Implant infection model in rats and rabbits	Konijnen (Oryctolagus cuniculus) / new sealand white	176	Matig
Spinal fusion in rats and rabbits in combination with ectopic implants	Ratten (Rattus norvegicus) / fisher 344	66	Matig
Spinal fusion in rats and rabbits in combination with ectopic implants	Konijnen (Oryctolagus cuniculus) / new zealand white	70	Matig

Algemene voorwaarden
Op grond van artikel 10a1 lid 2 van de Wod zijn aan een projectvergunning voorwaarden te stellen

1) De vergunning wordt verleend onder de voorwaarde dat eventuele go/no go beslissingen worden genomen met instemming van de IvD.

2) In artikel 10, lid 1a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of

omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning.
Indien bovenstaande zich voordoet dient aanvrager dit in overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

Weergave wet- en regelgeving

Dit project en wijzigingen
Volgens artikel 10c van de Wet op de dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister van Economische Zaken een ontheffing is verleend.

Verzorging
De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn.
In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving
In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef
Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade

zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13c volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13d is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.

[redacted]

Van: Info-zbo
Verzonden: dinsdag 19 april 2016 16:09
Aan: [redacted]
Onderwerp: terugkoppeling besluit aanvraag AVD115002016445

Geachte leden van DEC Utrecht,

Bij de CCD is een aanvraag tot projectvergunning ingediend waarover uw Dec advies heeft uitgebracht. Het betreft het project " Slimme coatings voor orthopedische implantaten" met aanvraag nummer AVD115002016445, uw interne code 2015.II.548.037.
Op basis van uw advies heeft de CCD besloten de aanvraag te vergunnen, aan de vergunning zijn twee algemene voorwaarden verbonden om te voldoen aan datgene wat voortkomt uit artikel 10a van de wet. De aanvrager is van het besluit op de hoogte gesteld.

De CCD dankt u voor het uitbrengen van uw advies,

Met vriendelijke groet, [redacted]

Centrale Commissie Dierproeven
www.centralecommissiedierproeven.nl
.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl (let op: nieuw emailadres!)