Fundamental consideration of Research: with reference to the AO Research Institute and the AO Foundation

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It is challenging and important alike to consider the aims, tasks and management of research. The first priority is to clarify what the aims and purposes of research in a given environment are. What sort of research work comes under consideration, how important are the working methods to a Foundation, which special requirements must be taken into account and which problems are to be reckoned with?

Purpose: The purpose of the AO Research Institute might be to distinguish the Foundation as a scientifically competent institution. Scientific reputation is important, but it cannot be the main or even the only research purpose of the AO Foundation. In contrast to academic research, AO Research has the clear duty to open up new approaches and to make the basic research carried out by the Foundation and Universities accessible in order to optimize the treatment of traumatized patients. Research should enable the Foundation to lead the field of trauma care of the locomotor system. The following specific tasks and work methods may be distinguished:

Scientific service: Researchers reply - on the basis of their expertise and resources - to practical questions arising in clinic and industry e.g. information on specific biomechanical issues.

Scientific projects: This work is highly structured in its purpose, method and schedule and culminates in scientific publications. It usually leads to incremental progress and indicates ways to optimize patient treatment e.g. experimental clarification of local infection resistance. Scientific projects at the fundamental research level take place in close collaboration with a network of internationally active institutions usually associated with university research. This work also facilitates academic promotion: many temporary collaborators of the AO Research Institute are today Professors and Doctors.

Scientific creativity. Creative, unconventional research and lateral thinking are the basis of scientific and clinical leadership that open the doors to unusual treatment methods for locomotor injury e.g. insight into the nature of temporary bone loss and its avoidance by application of the internal fixateur.

Classification into these three categories is not intended to reflect any form of rating but rather to show that different approaches require different environments, management strategies and communication. All three forms of research are essential.

The different levels of relevance of the aforementioned working methods for the AO Foundation are obvious:

Service: Service does not exist specifically to provide influence for the Foundation, and its contribution to the leadership requirements of the Foundation is limited, but yet service is indispensable. Scientific service is based on clear communication of the clinical problem and solution. Service management is conducted in accordance with standard procedures.

Projects: These consolidate the scientific reputation of the Foundation through subsequent teaching and publications. The nature of their purpose and the resultant knowledge help to solve problems and to compare different methods. Project work generally indicates a better way rather than a fundamentally new approach.

Creativity: Real and lasting leadership presupposes the ability to create, explore, validate and disseminate consequences of important new insights even if they are unusual and unexpected. Improving technology of teaching and public relations does not, by itself, result in lasting leadership if it is not supported by ongoing creative research in the lab and in the hospital. Long-term, high-risk research projects lead and avoid the trampled paths behind the herd. They cannot be directed administratively in the usual sense and are incompatible with the concept of early and frequent publication and extensive administrative activity.

Specific Requirements : Although project work is tightly structured in terms of aim, time and funding, it is imperative that part of the activity can respond as required to a change in circumstances or demands. It is essential in this kind of research to have a certain amount of freedom and flexibility with regard to the ultimate aims.

The opportunities, possibilities and frameworks for creative research are difficult for an outsider to understand, and cannot be considered and evaluated in terms of quantitative parameters. Due to the creative component, contributions from creative research are long-term and can and must not be considered as such in the regular, short-term

evaluations of the scientific performance of an institution. This type of research is very demanding in terms of directorship, goes beyond the normal scope of investigative endeavor and demands understanding and risk-taking from management. A prerequisite of creative research is a certain amount of freedom for the researcher and the research methodology. The risk that some projects will fail must be considered and accepted.

Evaluation of the quality of research deserves special attention. If the research cannot be adequately assessed within the institution, evaluation by the editorial board of scientific journals is a solution frequently chosen. Unfortunately, this procedure often only determines whether the methodologies fulfill prescribed technical requirements and the outcomes do not collide with the reviewers' existing knowledge. This level of review is hardly conducive to genuine progress in the sense of new insights. Evaluation of the value of creative scientific activity to the Foundation cannot really be delegated to authorities that are either unaware of the specific requirements of the Foundation or do not take them into account. Proper evaluation assumes a sound knowledge of the aims, function, requirements and expertise of the Foundation, and so the judicial selection of such experts as "auditors" is vital.

One aspect to be taken into consideration arises from the requirement that the researcher is able to experience clinical problems continuously at close hand so that he/she is well informed and motivated, i.e. the researcher has to be a member of the Foundations institutions that deals with problems in the clinic. The value of frequent direct contact and reciprocal visits involving clinicians is often underestimated.

In the thirty years of AO research, it was the challenging of generally accepted assumptions and the embracing of new insights that justified the leading role of the AO. In every area of research in Davos it was creative work that led to the development of new procedures, work which would have been given little opportunity in the context of service delivery or structured projects.

Unconventional thinking led to the strain theory of induction and repair tolerance and to the development of flexible osteosynthesis and the internal fixateur, opening up new territory and having a direct and important effect on clinical practice. Scrutiny of the histologically attractive mechanisms of primary bone healing and the mechanisms of necrosis also led to genuine clinical progress by stimulating and underpinning the concept of biological osteosynthesis. Concern for spontaneous tissue reactions as well as for the function of the injured limb and patient replaces or complements the rigidity of compression or thinking.

It is important that an integrated system of research and management of clinical outcomes develops that includes smooth transfer to an education program that ensures dissemination and sustainability.

Since a relatively large number of doctors active in trauma surgery worldwide do not perform internal fixation every day, an important demand on osteosynthesis technology is that it should not require the skills of the lonely expert. The techniques must not be rigid and demanding but "forgiving". The surgeon is confronted during the operation with particular, distinct biological issues and, in the face of limited exposure; the simplest technique is the safest. Demanding or, incompatible technologies in the hand of the non expert may jeopardize safety.

Assessing the value of unusual ideas is very demanding. It requires in depth discussions and it cannot be achieved by simple surveys. The introduction of the locking nail and the locking head screws of the internal fixateurs only long after their conceptualization and completion of their development are examples of an inherent stubbornness with regard to assuming that yesterday's procedures correspond to the "best proven technologies".

Focus has become a buzz word. In recent years, focusing on defined priorities such as osteoporosis has helped the AO Foundation to concentrate on central issues. Energy applied resolutely, flexibly and in moderation has been of genuine value in supporting the aims of the AO Foundation.

Research at the AO Institute offered unique opportunities to question and explore clinical fundamentals. Theories that make sense to us because they apply our reality (intelligence and logic) to structures and organs can be misleading. It is often overlooked that biological structures and organs possess neither intelligence nor logic. Does the bone know what we know? Does the bone want something? Can implants be intelligent?

Thus, the main emphasis and top priority of research at the AO Institute was the resolution of clinical problems and the improvement of fracture treatment. The aims and, almost logically, geographical location of the AO Research Institute were unique compared to those associated with university research, which acts, not a role model, but a necessary basis for and complement to AO activity. Buoyed by the acceptance and enthusiasm of AO members, AO research in Davos was able to develop to a large extent free from the academic pressure of "publish or perish". Rather than

publications, the criterion of success was the help offered to the patient and the doctor by innovative procedures. Success was measured by procedures that addressed burning clinical problems and that could be executed by the surgeon at large – fulfilling a philosophy that the simpler the solutions the safer they are.
It is vitally important (but not always easy!) to resist the enticement and temptation of fashionable ideas, concepts and trends (e.g. electro stimulation). Attractive new terms such as mechano-biology, biotechnology, stimulation of bone healing, tissue engineering etc. must be consistently evaluated in terms of their contribution to the AO Foundation and thus to the patient. AO research has a highly interesting, fascinating and rewarding role to play creatively challenging precepts and fundamental views.

Genuine progress cannot be called up, it originates from within.

Outlook: If we ask ourselves what the role of the AO Research Institute might be in the future, the following aspects stand out: The research laboratory in Davos is ideally suited to function as the central think tank for the AO Foundation. It should continue to support the foundations leadership through creative research whilst being ideally a pool of human and technical resources available on request according to the capabilities of the Foundation in all its diversity. In addition, the lab in Davos can continue to foster reciprocal communication and research coordination. Such coordinating and unifying functions are essential to provide focus in a world that tends towards diffusion and drifting apart.
How should these roles take form? Neither centrifugally, without fragmentation, nor centripetally, without patronizing. If every institution contributes best practice, if every contribution receives the appropriate recognition, then the collaboration will be genuine, spontaneous and, consequently, corresponding ideally with the ideas of the Foundation. In such a setting, the institution “Davos” can act as center of competence as primus inter pares without arrogance but as a natural and neutral point of reference. “Davos” is a worldwide renowned brand within the community trauma surgery.
The past thirty years were filled with fascinating activity and unusual research. I am convinced the AO Foundation’s coming decades will continue with productive engagements for the best care for the trauma patient. Everybody involved with the AO Foundation’s research institute in Davos could feel the creative buzz coming out of this worldwide unique place. I am deeply grateful for the circumstances and opportunities that brought me to the AO Research Institute and even more to all the people who participated in this endeavor creating the perfect research climate.

Locking Screw Implants in Internal Fixation

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INTRODUCTION: By late seventies and early eighties *in vivo* studies conducted at the AO Research Institute, Davos, under direction of Stephan Perren produced convincing evidence that early temporary porosis, commonly observed under the conventional bone plates was the result of the insult to the periosteal blood circulation caused by the implant-bone contact. Deprived of blood perfusion the bone turned necrotic, then sclerotic before undergoing remodelling through neovascularization, resulting in early, frequently persistent porosis. While in the majority of cases the end result was still a bony union, many of the major complications of internal fixation could be rationally linked to the vascular damage caused by the surgical intervention and the implants used. Infection tops the list. Could one improve by implant design?

METHODS: Two engineering proposals from the Straumann Institute, Waldenburg, provided the leads: (i) Brunner had proposed to increase DCP deformation tolerance in fatigue by transverse undercuts between the screw holes, evening out longitudinal variation in plate stiffness; (ii) Sutter had developed a mandibular reconstruction locking plate system. *In vivo* testing of the Brunner plate has provided some of the most convincing evidence linking bone remodelling to perfusion damage. Sutter's *in vitro* testing of the locked vs. conventional plates has demonstrated mechanical advantages of locked screws.

Combining those two proposals with a novel technique of locking the screw by means of friction between a conical head and a conical hole in the plate has initiated the development of a new plating system, PC-Fix, or Point Contact Fixator. The name was chosen to distance the system from conventional plating, which has, at the time, come under considerable pressure from the expanding indications for interlocked nailing.

RESULTS: Approximately eight years of testing on animals demonstrated some anticipated and some surprising advantages of PC-Fix when compared to conventional plating:

1. Significantly increased resistance to infection;
2. Faster, more consistent, healing;
3. Reduced impact on bone remodelling.

PC-Fix was then taken into clinical setting in both veterinary and human applications. A large, multi-centre clinical trial on forearm fractures largely met the expectations, but the system was not commercialized.

DISCUSSION & CONCLUSIONS: Elucidation of the role that vascular damage plays in plating for internal fixation has provided a fertile ground for innovation in what has appeared to be a near perfection in the art of implant and surgical instrument design, production and application. Commercial interests have also stimulated new developments – the patent on DCP, the single most defining product feature, a trademark of a sort of the AO and its commercial partners, was about to expire. A spur of activity in research, development and marketing in the eighties has resulted in the release of the LC-DCP system, featuring a modification in design and establishing titanium as the metal of choice, but the surgical principles of application remained the same. Unlike PC-Fix, *in vivo* testing of LC-DCP did not demonstrate any major advantage over DCP.

PC-Fix has been put aside, but the locking screw principle has caught the attention of both the industry and the surgical community and within a very short period of time it has become a standard feature on just about every internal fixation system. Technical solutions have also proliferated, but the main message of the research that has started it all seems to be still looking for the audience.

Kyon, a Zurich-based, veterinary surgical products company has entered the field as well, by releasing its Advanced Locking Plate System – ALPS – in 2007. The plate of the ALPS is a combination of Shermann (1907) and Brunner plates, with its holes providing for use of either conventional or locking screws. The shape of the plate allows for bending in both planes – FE analysis suggests superior mechanical properties in comparison with the similarly sized DCP. The material is exclusively commercially pure. Titanium (cpTi). The screws are either cpTi or titanium alloy. Three sizes suitable for small animals are currently available. The first clinical reports from a limited number of veterinary surgeons who have used ALPS for now a year are expected in the fall of this year.

Locking Screw Fixation for Total Hip Prosthesis

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INTRODUCTION: While the general consensus may be that hip replacement surgery is very successful, the Swedish National Hip Register[1], which has been tracking all hip replacement surgery performed in Sweden since 1979, presents a different picture. The Register shows that the revision rate for non-cemented stems over all age groups is approximately 17.5% at 10 years. Furthermore, when looking at only the age group of patients less than 50 years of age, the revision rate at 10 years increases to almost 25%. From their data it is estimated that there is a worldwide revision burden of between 20 to 25%.

It is well established that a key to long term success of total joint replacement implants is early stabilization. Some stem designs utilize screws to provide additional stabilization of the stem to the bone. These screws are similar to those used with interlocked nails. However, these constructs are prone to deformation under load, fretting, and mechanical failure.

Locking screw fixation of trauma plates, called internal fixators, introduced in the late 80's brought many advantages to orthopaedics. Iatrogenic bone damage was reduced, resistance to infection was increased, fretting corrosion was practically eliminated, and the plate/screw constructs remained stable and intact. Application of the locking screw principles to intramedullary anchorage of joint replacement components was a logical extension from internal fixation.

Locking screw fixation of total hip replacement stems has been successfully demonstrated in over 15 years of clinical experience in over 7500 dogs.

METHODS: A short curved stem was chosen for the design to reduce loss of proximal femur cancellous bone, to allow one stem shape to fit both left and right sides, and to facilitate the surgical approach. The medial endosteal contour of the proximal femur was determined by averaging contours from 75 A-P x-rays corrected for ante version of the neck. The length and final shape were arrived at through iterative FE design, handling studies in cadavers, and trialing during clinical procedures.

Stresses in the stem, screws and the bone were calculated using FE analysis with the applied load

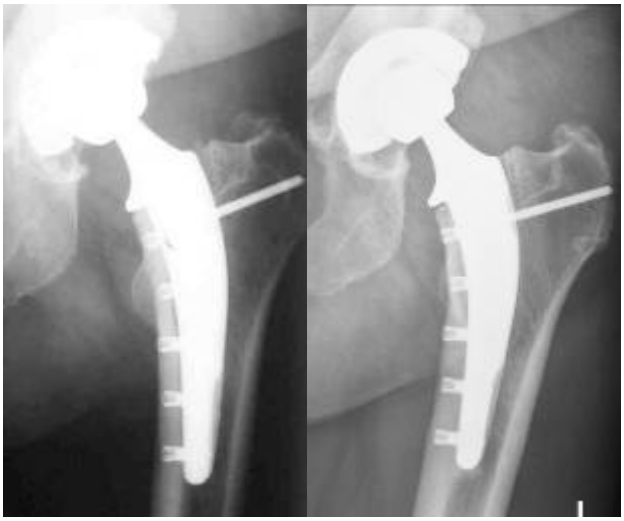

Fig. 1: AP x-rays of 53 year old female shown with a locking screw fixation stem l)immediate post op r) 14 months post op.

defined according to ISO 7206-4, -8. Fine tuning of the stem shape was achieved through iterative FE analysis. A collar, lateral recesses, extension of the proximal screw and neck transition sections were added/modified in order to reduce critical stresses in either the implant or the bone.

Fatigue testing of the stems according to ISO 7206-4, -8 was performed (Fraunhofer Institute, Freiburg, Germany) using both the old fixation level (more demanding) and the new level. Testing was also performed with the screw holes left empty to assess the risk of stem failure due to surgical error. A final series of tests were performed using mechanically equivalent femurs constructed from glass/epoxy (Sawbones).

RESULTS: The stem successfully passed all series of fatigue testing, even with the screw holes left empty.

DISCUSSION & CONCLUSIONS: Clinical follow-up data from use of a similar design in dogs has shown only 0.65% aseptic loosening of the stem in 460 consecutive cases[2]. As anticipated by the results in dogs, initial clinical results in human show bone apposition toward the medial surface of the implant and no remodeling of the lateral cortex.

REFERENCES: [1] *Swedish National Hip Register*. [2] A.Vezzoni, BVOS, Birmingham,UK,Apr. 2008.

Tissue Sparing Hip Implants

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INTRODUCTION: Hip joint replacement is an extremely successful procedure, with failure rates of less than 10% at 10 years reported in national registers. Particular stems are shown to perform even better than this. However, small proportions still relate to large numbers, which are continually increasing, particularly the proportion of younger patients. In these cases, implants must last longer, under harsher loading conditions.

On the femoral side there is a trend towards uncemented implants without the traditional diaphyseal shaft. The latter has a number of potential advantages: One is more physiological loading of femur, which can reduce the phenomenon of "stress shielding" and consequent bone loss. Another is the possibility for less invasive insertion and decreased disruption of both hard and soft tissues.

The potential disadvantages of removing the distal stem are loss of implant stability. The stem must be sufficiently stable to allow bony ingrowth and must distribute loads so as to prevent bone overload. To maximise the chance of safe mechanical integration of such implants preclinical simulation and testing is necessary. Furthermore, careful monitoring of clinical failures is invaluable.

In this paper a number of examples of such "proximal femoral implants" is introduced and their clinical testing is described. An example of a retrieval study is also presented.

METHODS: Various modern implants, all anchored in the proximal femur, have been tested for stability. They have been subjected to mathematical analysis and experimental testing to investigate potential implant stability in bone. Mathematical models were developed with friction simulated at the implant-bone interface, allowing relative motion to be estimated under joint loading.

Implants have also been tested for stability by implantation in cadaveric bone. Simulated joint loading was applied and relative displacement between stem and bone was measured using an optical system, with markers mounted to each component.

Finally, a retrieval study is described in which over 250 failed "resurfacing" implants were collected from clinics internationally and examined for failure mechanisms.

RESULTS: Mathematical models indicated that the proximal femoral implants analysed could lead to low relative motion between the stem and bone. It was also demonstrated that bone loads increase with neck offset and varus implantation.

The experimental studies indicated that relative motion was less than for conventional "stemmed" implants and that strength was not compromised by the shorter shaft. Furthermore, it was shown that there was greater flexion of the bone for the proximal stems. However, stems with high neck offset led to failure of the medial Calcar.

The retrieval study showed that more than two thirds of resurfacing failures resulted from fracture of the femoral neck. These occurred within the first year after surgery.

DISCUSSION & CONCLUSIONS:

Proximal femoral implants provide a new generation of solutions for an ever expanding number of patients, including a more demanding group that are both younger and more active. They can better preserve soft and hard tissues by being smaller than conventional stems but also demonstrate potential for integration in the bone, based on low relative motion between stem and bone. This has been demonstrated both in mathematical models and experimentally. Furthermore, the greater deformation of the femur demonstrated shorter stems indicates that there may be less chance of stress shielding and bone loss for such implants.

At the same time, it should be noted that such proximal implants may be less forgiving regarding implantation, and demonstrate a distinct learning curve. Damage to the femoral neck during reaming has been blamed for fracture. Varus placement and high neck offset increase the moment arm and lead to higher bone loads, which can be particularly dangerous in bone of poor quality.

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Locking compression plates (LCP) & less invasive stabilization system (LISS).

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The development of the less invasive stabilization system (LISS) for the management of distal femoral fractures and proximal lateral tibial fractures was the technical answer to the clinical need of a less invasive plate osteosynthesis. The LISS is an anatomically pre-shaped internal fixator which can be inserted percutaneously by means of an adaptable insertion guide. In combination with a trocar assembly, the handle also serves as an aiming instrument for an exact percutaneous placement of screws. Based on extensive anatomical studies, the orientation of the individual screws is predetermined. As the LISS plate is anatomically pre-shaped, small variations between the plate shape and the anatomical topography can be encountered. To compensate these variations, special locking head screws have been developed. A conical outer thread of the screw head and an inner thread of the plate hole, locks the screw into the plate, as soon as the threaded screw head engages the threaded plate hole and the screw are tightened together. The locking head screw locks the distance between the bone and the plate as well as the screw axis relative to the plate-hole axis (angular stability). To facilitate the placement of screws trough small skin incisions, the LISS locking head screws are self tapping and self drilling. The self drilling feature can only be implemented, while the screws are place uni-cortically.

In contrast to any previous osteosynthesis plate, the LISS plate is forged out of a titanium alloy and stainless steel, to improve cyclic load to failure. Again this production process can only be used, when the plats are not shaped by the surgeon in situ.

The LISS for the distal femur (LISS DF) and the proximal lateral tibia (LISS PLT) are implants that act as splints. The LISS acts mechanically as an internal fixator. It is a 100% locked internal fixator, because only locking head screws (LHS) are used. The LISS is designed for percutaneous insertion. A less invasive approach is also possible. Important is a closed, indirect reduction and a pur splinting of the fracture zone.

The development of the locking compression plate (LCP)

The LISS was originally designed as a device that would only accommodate locking head screws; because all of the plate holes are threaded. However, clinicians found that this technology was too restricted in some cases and that an all-purpose implant system would offer greater flexibility. Development work in this area, with multidisciplinary collaboration among clinicians, developers and manufacturing engineers led to the concept of a combination hole, combining the use of compression –and locking head screws in one hole configuration.

The LCP hole also makes it possible to insert different screw types into the same plate, so that the surgeon is able to choose the type depending on intraoperative requirements. In retrospect, combining two completely different anchorage techniques into a single implant was a logical approach and a straightforward, practical solution. With the LCP, the surgeon has two plating methods to choose from and is able to select the more appropriate of the two techniques. With its newly designed combination hole, the LCP makes it possible to implement the principles of both compression and splinting for fracture stabilization in the same implant. The option of using the LCP either as a compression plate or as an internal fixator, provides ideal plate anchorage that can be adapted to requirements of the individual case. This significantly extends the range of indications in minimal invasive plate osteosynthesis.

Femeroacetabular Impingement

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INTRODUCTION: The pathomechanism of hip pain and hip osteoarthritis is multi-factorial. One probable mechanism is femoro-acetabular impingement. “Cam” impingement results from abnormalities on the femur, in the form of a non-spherical femoral head or a protuberance extending from the head towards the femoral neck. When the femur flexes, this contacts the labrum on the acetabular rim, impinges and eventually enters the acetabulum, deforming the acetabular cartilage. The “pincer” type impingement occurs due to over-coverage of the femoral head by a deep acetabulum. In this case, the normal femoral neck impinges against the labrum during flexion, compressing and deforming the labrum. In contrast to the pincer type joint, the dysplastic pathological joint is the result of an insufficient coverage of the femur head, which leads to high pressures across the small acetabular roof. Joint preservation surgery is an option to restore normal hip geometry. A comprehensive knowledge of hip function and the stress distribution inside the normal and pathological joints can help to identify critical regions and provide a scientific basis for surgical planning. The goal of our ongoing work is to simulate the influence of hip joint morphology on the internal mechanical environment of the joint, to evaluate the hypothesis that impingement or dysplasia may be biomechanical precursors to joint degeneration.

METHODS: The geometries of normal and pathological joints were simulated, based on standardized morphometric measures. The most appropriate parameter to define the morphology of a cam type pathological joint is the alpha (α) angle, defined as the angle between the line from the head center to the center of narrowest part of the neck, and the line connecting the head center to point at which the femoral head becomes aspherical. For the normal joint, this is approximately 40°, but for pathological joints may vary between 60° - 90°. The center-edge (CE) angle is defined as the angle in the frontal plane between the vertical line through the femoral head center and the line from the center of the femoral head to the acetabular rim. A normal CE angle is about 25°. A CE angle of less than 15° is associated with the dysplastic hip and a CE angle of greater than 35° is characteristic of a pincer type joint. Joint models were developed with CE angles of 40°, 35°, 30°, 25°, 20°, 15°, 10°, 5°, and 0°. The cam type joints were developed with alpha angles of 80°, 70°, 60° and 50°. The diameter of the femoral head was 50mm, with a cartilage thickness of 2 mm. Loading and kinematic data for daily activities of stance, walking and sitting down were derived from in vivo measurements. The material properties were extracted from literature values.

RESULTS: It was observed that changing the CE angle results in a shift of the regions of high shear stress within the cartilage layers. For low CE angles, a zone of high shear stress occurs near the acetabular rim, where labrum hypertrophy and calcification occurs for dysplastic joints. For large CE angles increasing to normal (about 25°), the area of high shear stress is reduced, as well as peak stress values. Increasing the CE angle to 40° leads to high shear stresses in the antero-lateral portion of the acetabulum, where the acetabular cartilage connects to the labrum. During motion, regions of high shear stresses are observed for both the cam and pincer joints, initiating at the labrum where it is being impinged and spreading to the acetabular cartilage, at the interface where it connects to the labrum.

DISCUSSION & CONCLUSIONS: Regions of high intra-articular shear stresses observed for low CE angle (i.e. dysplasia) and for impingement cases are relevant and correspond with clinical findings of region-specific cartilage delamination or calcification. This study highlights a safe range for the alpha and CE angles, in which the hip joint can function without a heterogeneous articular loading or local cartilage stress concentrations. The results of such simulations can provide guidance for the preoperative planning of joint preservation surgery, to determine target values for post-operative joint morphology.

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Understanding bone healing: combining *in silico* and *in vivo* approaches

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INTRODUCTION: Most bone fractures today are well treated with current implants and heal uneventfully. However, complications do persists, e.g. 5-10% of all fractures in humans do not heal in a timely manner [1]. Furthermore, incidence of delayed unions may increase due to the aging population and diminished regenerative capacity of the elderly. Clinical factors such as general health, trauma severity, surgery, fixation and post-operative care all interact in determining the biological and biomechanical conditions of the healing fracture which in turn then determines if it will heal in a timely manner, if at all. Thus, in order to rationally develop treatment strategies to overcome delayed- or non-unions, we first need to understand this complex interaction involved in the healing process.

Secondary or indirect bone healing is a regenerative process involving both intramembranous and endochondral ossification. During ossification, progenitor cells become activated, proliferate, apoptose, migrate and differentiate into various cell phenotypes. Mature cells also react similarly as well as synthesize and remodel their tissue specific matrices. In all of these processes, the active component is the cell which is known to be sensitive to its physical environment.

A decade ago, Prendergast and Huiskes first proposed that mesenchymal progenitor cell differentiation can be mechano-regulated by the local combined magnitude of interstitial fluid flow velocity and tissue shear strain [2]. We hypothesize that this mechanoregulation principal is also applicable to fracture healing. Our approach to demonstrate this has been to develop a computational model, based on this principal and established cell based processes, capable of simulating the biological process of indirect fracture healing and determine if this model could also be corroborated again *in vivo* experiments of abnormal fracture or bone healing.

MODEL: First a 3D finite element model (FEM) of an idealized mid-diaphyseal fracture in an ovine tibia reduced with a small gap was developed. The marrow, cortical bone and an initial callus of granulation tissue was modelled with poroelastic elements and material properties from the literature. This FEM was then embedded in another model where cellular processes were quantitatively described with partial differential equations, logical rules and parameter values derived from *in vitro* and *in vivo* experiments in the literature [3].

RESULTS: The model was able to correctly simulate the temporal and spatial distribution of tissues during normal fracture healing under axial loading. It was also able to simulate delayed and non-union formation with overloading as observed by Claes et al. [4] as well as other abnormal healing conditions such as delayed healing due to periosteal damage and genetic deficiencies in cartilage resorption [5-6].

To challenge the model it was also corroborated against *in vivo* experimental conditions of pure torsional interfragmentary motion. Similar to the *in vivo* results, the model simulated a unique spatial distribution of intercortical gap bone formation. Finally, the underlying model was adapted to simulate distraction osteogenesis and compared to an *in vivo* experiment in sheep [7]. The model was not only able to simulate the experimental results but also to correctly capture the affect of distraction rate and frequency on bone formation.

CONCLUSIONS: These comparisons of *in silico* and *in vivo* results support the validity of the mechanoregulation theory first proposed by Prendergast and Huiskes and suggest that *in silico* models of fracture healing may be ready to be used for implant design and in the clinics in the near future.

REFERENCES: [1]A. Praemer, et al (1992) AAOS. [2]P. Prendergast et al (1997) *J Biomech*. [3]H. Isaksson et al (2008) *J Theor Biol*. [4]L. Claes et al (1997) *J Orthop Res*. [5]C. Colnot et al (2003) *Develop*. [6]N. Kosaki et al (2007) *Biochem Biophys Res Commun*. [7]U.H. Brunner et al (1994) *Clin Orthop Rel Res*.

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Carbon Fibre reinforced PEEK medical Implants

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CFM Technology. The Composite Flow Moulding (CFM) process is a single step processing technology that has set new standards in the use of composites. The net-shape technology enables the manufacturing of fastening elements and other load-bearing components made of continuous fibre reinforced thermoplastics. Pultruded rods with an endless fiber content of approximately 60 % by volume and a thermoplastic matrix are heated above the melting temperature. The products are formed by pushing the material into a cavity, while controlling temperature, pressure and progress. The process allows for shaping complex forms while leaving the continuous fibres intact. This results in components with excellent properties with respect to fatigue resistance, flexural strength, shear and tensile strength. A wide range of different fibres and thermoplastics can be processed using the CFM technology, thus enabling application specific material combinations. The implementation of this CFM technology into the medical field using continuous carbon fibres and PEEK as matrix (CF/PEEK) initiated the development of orthopedic implants with outstanding fatigue properties and optimized visualization in various imaging technologies. icotec ag is the industrial realization of this CFM technology that has been developed since 1992 in cooperation with the Chair of Biocompatible Materials Science and Engineering at the Swiss Federal Institute of Technology (ETH Zürich).

General application. High strength composites of various fibres and matrices have a wide range of applications using combinations of the specific advantages. CFM parts are used in industrial application in areas like fast moving parts, aeronautics, sports, racing and some special applications in research where e.g. metals are totally banned.

Medical application. icotec implants are mostly based on ENDOLIGN™ from Invibio. This medical grade material (CF/PEEK) is used for short- and long-term implantations. This material processed with the icotec CFM technology resulted in a number of innovative CF/PEEK implants like the intervertebral spacers, translaminar facet screws, cervical plates and other implants in orthopedics and traumatology.

Mechanical properties. In general the CF/PEEK as used by icotec is one of the composites with the highest mechanical properties available. Fatigue properties similar to titanium and static strength as with aluminum make it possible to develop products with true weight bearing functions. In an in vivo handling study (tibia osteotomy in sheep) the icotec Snakeplate and a titanium traumatology plate were compared. The callus formation over time and the mechanical quality of the operated tibiae after 8 weeks were equal, indicating good bone osteotomy healing. These results were repeated in a clinical trial treating humerus shaft fractures.

Material modulus. The modulus of the material can be adapted to the product requirements by adjusting the fibre orientation in the implants. In general the modulus can be expected in a range from 30 to 60 MPa. This is much closer to the modulus of bone than for example titanium (>100). The material modulus combined with the fatigue properties makes it possible to develop dynamic implants. Dynamic implants might promote fracture healing or support physiological functioning of structures.

Biocompatibility. The material CF/PEEK is inert causing a non-specific tissue reaction after implantation. In a comparative in vivo study the soft tissue reaction and reaction to debris did not show significant differences to tissue reactions to Titanium implants. The bio-compatibility of icotec implants is excellent and comparable to PEEK implants. To improve bone-implant contact, if required, a range of coatings are available. In vivo studies showed excellent binding between CF/PEEK, the coatings and newly formed bone.

Optimal visualisation of implants. Composite implants with CF/PEEK are radiolucent and, though visible in CT and MRI, do not cause artifacts as metal implants do. These properties allow for better observation of the region of interest and the use of radio-therapies. icotec has an unique patented procedure to visualize the implants. If required, a controlled visibility can be achieved by adding tantalum fibres. The unique combination of these properties allows icotec to use CF/PEEK for implants in spine surgery, orthopedics and traumatology applications.

The role of implant surfaces in fracture fixation

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The development of a stable bone/implant interface (though not necessarily direct osseointegration) for partial or complete stability of an implant device is critical for the success of osteosynthesis implants, such as screws, pins and nails. Bony integration is increased on implant surfaces with increased roughness, though really this is only within a range of 200nm to 2µm. We believe osteoblast cells do not react to roughnesses outside this range. The majority of research for surfaces throughout the world has been towards increasing bony integration. This trend is probably inappropriate for internal fracture fixation (IFF), apart from in special areas such as in spine fusion and long term or permanent CMF implants.

IFF devices are often removed to avoid: growth disturbances in paediatrics, allergic reactions, soft tissue irritation (prevention of tendon gliding within hands), implant protrusion/intrusion (e.g. into a joint), pain experienced by the patient or being cosmetically disturbing (protrusion under skin or even optically disturbing), implant migration/breakage, build up of fretting particles in unrelated organs (from loose multi-component implants), in cases where infection of the device or adjacent tissue has occurred as well as due to cold intolerance of the patient. There are difficulties in removing a device. These include increased operative time due to difficult removal of strongly integrated bone to screw threads, screw heads and even the plate itself, screw stripping and breakage. This has caused industry to have developed special removal devices to retrieve these parts. These problems also have associated risks such as increased blood loss and debris contamination.

In temporary implants such as plates and nails with the use of screws or the application of external fixators, minimal direct bone bonding to implants is desirable for the least traumatic device explantation. Strong bony integration is a disadvantage when the device may later need removal. Surface microtopography is the major determinant of bony integration for current clinically used metals.

Surface polishing reduces microdiscontinuities (Ra 0.2 to 2 µm) that can be ‘seen’ by the cells producing surfaces of low roughness (Ra less than 0.2 µm). Our *in vitro* work with osteoblasts has shown that polishing acts on a cellular level (as it does with fibroblasts). Implant surface topography influences osteoblast differentiation, reducing expression and function of genes specific for osteoblast phenotype, compared to standard micro-rough counterparts. Polishing had a strong effect on one gene osteocalcin, significantly reducing its expression. This surface induced cell behaviour change is achieved initially due to the surface altering the cell shape/cytoskeletal organisation.

Our *in vivo* study in rabbits in the late 90’s showed that increasing the surface roughness of EPSS (electropolished stainless steel) internal fixation plates when roughened (Ra 0.2 to 2 µm) induced more bone formation towards the implant surface without fibrous tissue formation in between, compared to plates outside this range. These results support the hypothesis that bony integration is increased on implant surfaces with higher amounts of protruding microdiscontinuities that the cell can ‘see’. More recent *in vivo* work within the group assessed the effect of surface topography of TAN and cpTi screws with different surface topographies (polished and microrough) in a sheep cortical (tibial) and cancellous (rib) bone model over three time periods of 6, 12, and 18 weeks. The effect of implant topography on bone adherence was evaluated mechanically by measurement of the peak torque removal force and histological assessment of the amount of bone present at the surface of the implant. The results demonstrated that polishing both cpTi, and TAN resulted in lower removal torque than standard microrough screws when placed into cancellous and cortical bone.

In a more recent *in vivo* study we compared the pull out forces required for the removal of standard TAN (NS), and EPSS IM nails, and for the removal of NS and paste polished TAN (PP-TAN) IM nails, from bilateral, non-fracture sheep tibia model, after a 12 month implantation. This novel study successfully demonstrated the effect of implant surface

polishing on reducing pullout force for intra-medullary nails. Since TAN is preferred over SS for IM nailing due to its better biocompatibility and mechanical properties, we believe these findings could be used to recommend changes to current surface technologies of IM nails, to reduce complications seen with nail removal, especially in rapidly growing bone in paediatrics.

For bone fixation plates such as the LCP and LISS, we have identified excessive bony on-growth to the plates and in-growth within the screw holes as a major contributor to removal failure. Some studies suggest early removal once a fracture has healed, in an attempt to circumvent this problem, however, by the time fracture healing has occurred, encasement of the implant by bone, may also have occurred. Additionally, premature removal of an implant can jeopardise the healing of a fracture. Therefore in another *in vivo* study we reduced the surface micro-topography of clinically available LCP's, with locking screws. Our findings showed that surface polishing significantly reduced the force required for removal of TAN cortical screws compared to standard micro-rough counterparts. Furthermore, there was a trend for lower percentage of bone contact on the polished samples compared to micro-rough screws; however, this was always significantly higher than that observed for EPSS screws. Moreover, the significant reduction in time required for tissue removal from polished devices, will directly reduce the surgical time associated with implant removal, thus improving, not only the economic burden associated with surgical procedures, but also the surgical related complications with regards to the patient, which are both principal deciding factors for implant removal. Consequently, we suggest that surface polishing is a promising technique for fixation devices destined for removal which would positively influence the prevalence of implant removal-related complications.

On the other side, implant loosening is an unresolved complication associated with prosthetics such as spine cages, where osseointegration is essential to their success.

Anodic Plasma-chemical (APC) treatment binds calcium and phosphate direct into the metal surface to produce superior adhesive strength than a coating, thus offering great potential for enhancing integration into surrounding hard tissue, while negating adhesion issues associated with conventional HA coatings. We showed APC to be both cytocompatible *in vitro* and biocompatibile and osteoconductive *in vivo*. The APC treated samples had similar biological performance to HA coated screws, though they had superior binding strength compared to standard HA coatings.

Polyetheretherketone (PEEK) has due to its radiolucent properties come into the spotlight as a replacement for metals in devices such as spine cages and craniomaxillofacial (CMF) implants. However, cellular attachment to PEEK is restricted due to its low surface energy, which can lead to fibrous encapsulation. To aid tissue integration the surface energy has been increased by plasma surface treatment incorporating oxygen into the surface. Surface treatment of PEEK has led to higher levels of nodule formation indicating that these treated surfaces are likely to improve bony integration to implants, though more detailed work is being undertaken.

In situations with either hard or soft tissue interactions with biocompatible bulk materials, the 'implant biocompatibility' is determined more by the design and surface characteristics. Without surface modification an implant may be biocompatible in one anatomical situation, (e.g. a spine cage surface increasing osseointegration) yet not in another (the same surface would be deleterious to free gliding of tendons). There is no `generic wonder surface` for all applications and surfaces even on one implant interacting with different tissues need to be considered as separate entities.

ACKNOWLEDGEMENTS: Thanks to my group (present and past) who worked within this area – Osian Meredith, Jessica Hayes, Joanne Welton, Pamela Furlong, Alexandra Pearce, Louise Hughes, Annika Persson, Alexandra Poulsson, Patrick Schlegel, Mauro Stifannic, Llinos Harris, Gethin Owen.

BIODEGRADABLE MATERIALS FOR OSTEOSYNTHESIS AND TISSUE ENGINEERING

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INTRODUCTION: The development of materials for osteosynthesis has been co-continuous with the improvement of the bone fixation knowledge and the sophistication of surgical procedures. Thus, thanks to their outstanding mechanical properties, metal devices made of titanium alloys are the gold standard for the majority of the fracture fixation treatments. However, they have several significant drawbacks. First, after the fracture healing, a second operation is often necessary to remove the implants and this has several risks such as infection, removal problems of jammed implants, implants migration and associated extra health care costs. Secondly, metal devices cause magnetic resonance imaging artefacts. Finally, the high modulus of elasticity of metals compared to bone, results in the implant retaining a large fraction of the mechanical load applied to the bone. This is known as the "stress shielding" effect which leads to bone resorption. As a result, it has been thought for a longtime that a material that will resorb and which progressive mechanical properties loss could match bone healing, would improve the final outcome of fracture surgery. The quest of such material has been the initial driving force behind the research on resorbable materials for osteosynthesis, especially biodegradable polymers.[1]

BIODEGRADABLE POLYMERS:

Poly(α-hydroxyacids) are still the more commonly used resorbable polymers for osteosynthesis devices. They are successful in non-load bearing applications, but their actual mechanical properties and design have not yet permit the widespread of their use. This report aims to emphasize how the actual resorbable devices properties and the lack of controlled randomized prospective trials that document their efficacy in treating a particular fracture patterns are partly responsible for the current limited use of resorbable osteosynthesis devices. Meanwhile, with the implementation of the recent gained knowledge and the better understanding of the biological mechanisms and factors influencing the living tissues regeneration, these devices could be "active" rather than "passive". Potential directions for the improvement of resorbable osteosynthesis devices and examples of the next generation of bioresorbable polymeric implants, notably resorbable hydrogel and polyurethanes carriers for tissue engineering constructs, are presented.[2] The improved control of the spatial and the temporal interfaces between the resorbable polymers and the surrounding biological tissues and cellular components is leading to hope for new cells based therapies.[3]

REFERENCES:

[1] S. Gogolewski (2000) *Injury* **31**(S4):28-32.

[2] S. Grad, L. Kupcsik, K.I. Gorna, S. Gogolewski and M. Alini (2003) *Biomaterials* **24**:5163-71.

[3] D.W. Hutmacher (2000) *Biomaterials* **21**:2529-43.

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STEM CELLS FOR MUSCULOSKELETAL REGENERATION

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The use of human stem cells to regenerate damaged tissue within the musculoskeletal system has been already applied to treat several of them, such as bone and intervertebral disc. Mesenchymal stem cell based approaches for treatment of other tissues, like cartilage, meniscus, ligament and tendon are at the later stages of development.

Although, some encouraging results have been observed, the clinical application of mesenchymal stem cells is far from being a routine approach in current medical treatments. Several biological and clinical issues have still to be improved. Presently, we are tackling two major problems, one related to the heterogeneity of mesenchymal stem cell isolation procedures and the second, which is more clinically relevant, concern the use of mesenchymal stem cells for repairing large bone defect. Mesenchymal stem cells are normally isolated by adherence to plastic or by using antibodies that recognize specific cell surface molecules (i.e. CD 90). With the first approach, other cells will also adhere to the well of the plate and indeed only few percent of all the adherent cells could be considered mesenchymal stem cells. The use of a CD antibody is also confronted with problems related to the specificity of the antibody, when used to fish out a homogeneous cell population from a heterogeneous mixture of phenotypically different cells. In order to improve the isolation of committed (osteogenic or chondrogenic) mesenchymal stem cells, we have developed a GFP-vector that once transduced into the cells (Adenovirus) will produce green fluorescence upon differentiation towards a specific phenotype (osteogenic or chondrogenic). So, we will be able to isolate those committed cells and further study their behavior. We could test if a more committed and homogeneous cells population would be more efficient (to form bone or cartilage) than those presently used, which are dispersed and fill the presence of different type of cells. Autologous bone grafting is the current golden standard for the repair of large bone defects, despite drawbacks such as limited availability of grafting material and donor site morbidity. Possible alternatives like allografts or xenografts also have serious limitations; the risk of infections, possible immune reactions and ethical issues. Due to these problems, researchers in the area of bone repair have explored alternative solutions. Calcium and phosphate based materials as well as polymer scaffolds have shown some interesting osteoconductive properties. Nevertheless, the lack of osteoinductive potential prevents the healing of large bone defects treated only with such alloplastic materials. Many studies have shown that the lack of osteoinductive potential of such scaffolds can be partly overcome by seeding mesenchymal stem cells (MSC) onto the scaffold prior to implantation. However, a major problem still remains, namely the insufficient vascularization of the central part of these large grafts (>4cm). Thus, one of the most limiting aspects in obtaining tissue-engineered bone suitable for repairing large bone defects is the inadequate bone vascularisation. We have therefore addressed the enhancement of endothelial progenitor cells (EPC) as one of the key mechanisms in autologous bone grafting. The means by which these progenitors for neovascularisation can be isolated and characterized have recently been described. However, one of the major obstacles preventing the clinical application is the time needed to expand the EPC in vitro in order to obtain the required cell numbers. Out of the hundreds of common culture media compositions specifically designed to effectively culture cells of endothelial lineage none seems to be powerful enough for the desired purpose. Furthermore, these media are not autologous, which would be ideal for clinical use. We have therefore investigated the “Platelet-released growth factors” (PRGF) cocktail as a possible autologous source for EPC expansion. Our results show that PRGF is a highly efficient growth medium for EPC in vitro expansion. Moreover PRGF maintains the endothelial differentiation capacity of EPC. Immunostaining and PCR analysis showed persistence of angiogenic markers on CD34+ and CD133+ cells up to 21 days of culture. In addition, the capacity to form a cellular network after expansion in PRGF medium indicates that the EPC/PRGF association could have a positive influence on the formation of a vascular network within bone tissue engineered constructs.

Current problems in fracture treatment: what the surgeon wants

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Results of fracture treatment have continuously improved since the AO started to study and to teach operative fracture treatment 50 years ago. During the last decade this evolution was characterized by the development of new intramedullary nails and locking screw-plate constructs which can be applied with less damage to the blood supply of the bone and which also allow safer fixation in osteopenic bone. Today there are plates and nails available specifically designed for almost any given fracture of the skeleton. Despite the impressive improvement of our armamentarium there remain however still some challenges for the surgeon.

Fracture fixation in osteoporotic bone: Osteoporotic bone remains a problem despite the new implants mentioned above. These implants initially are getting much better purchase in the bone. However, since the constructs are quite stiff they tend to cut through the bone in severe osteoporosis. This could be solved with fixation devices with a "softer" interface between implant and bone, most likely manufactured of materials or composites other than metals currently used. Another approach to avoid cutting through would be augmentation of the bone. The only material used for this purpose is currently PMMA which however is far from being ideal. The surgeon would wish a material that can be applied almost immediately, that augments a big volume of bone without the need to first create a cavity, that can be kept on the back table for hours and still "freezes" in seconds after its application or - even better - at the moment the surgeon wishes. It should however not do any harm, neither in the bone nor if it leaks out of the fracture site or into a joint. Of course it should dissolve over reasonable time without however leaving a void but healthy bone. At the end it should be affordable particularly because of the still increasing number of elderly patients.

Non-unions: Classic teaching tells that non-unions are either the result of lacking stability at the fracture site or of impaired blood supply of the bone. In many cases however the reason remains unclear. Fracture healing can be enhanced with locally applied BMP's or other mediators. If a delayed union is noticed or a non-union established there are many therapeutic options on the marked of which the proven efficacy is unclear. Research in this field should therefore answer the question which patients are prone to develop a non-union. Patients at risk should then get some sort of prophylactic treatment. Research should also focus on the different modes of non-surgical treatment of delayed and non-unions. One of the interesting questions in this regard is what makes fibrous tissue in a non-union transform into bone.

Large bone defects: Bone loss occurs after debridement of open fractures, resection of bone for osteitis or tumors. Segmental bone loss of some centimeters in diaphyseal bone inevitably results in non-unions. However, in addition to the treatment of the non-union the missing bone needs to be replaced. The surgeon would wish a product with which the defect could be bridged or filled immediately. Ideally this "bone filler" would have the same mechanical properties as bone. It would not only fill the defect, but lead to new bone formation and eventually be replaced completely by new diaphyseal bone. The new product should be ready to use immediately and storage should be simple.

Bone infections: Despite new antibiotics, better antisepsis, more aggressive debridement in open fractures and less invasive operative techniques for fracture fixation, infections and particularly implant related infections of the bone remain a unsolved problem. There exists no reliable method to early diagnose or rule out infection. In the treatment the mainstay today is debridement. Here the problem is to differentiate living from dead tissue particularly in the bone. Debridement leaves defects. Thus other treatment methods would be desirable which would cure the infection without resection. Contaminated implants, particularly with bacteria that form biofilms can not be cleaned. Therefore methods to sterilize such implants in situ without damaging the surrounding tissues would be a huge step particularly in joint prosthesis. Prevention would be even better. In this regard implants that are more resistant to infections or local antiseptic substances would help. The methods or substances used should however avoid the selection of resistant germs.

Conclusion: To address the current needs of the clinician research has to change its focus almost completely from traditional biomechanics and new implant design to a variety of new fields.

Osteoporosis: Who is Guilty?

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The modern human skeleton represents an end point of million years of ongoing adaptation. The skeleton integrates several vital non-mechanical functions (mineral homeostasis, hematopoiesis) in conjunction with its primary purpose – locomotion - into a single organ.

Sex steroids, especially estrogen, play an important role in skeletal homeostasis[1]. Estrogen is considered a bone conserving hormone[2] and its principal skeletal effect at the tissue level is suppression of bone turnover and maintenance of balanced rates of bone formation and bone resorption. Consequently, the loss of estrogen function at menopause is associated with a marked increase in the rate of bone remodeling and a negative balance between bone formation and bone resorption at the level of the bone multicellular unit (BMU) leading to bone loss. While the extraskeletal effects of estrogen (e.g., hypertrophy of the reproductive organs at puberty and atrophy at menopause) are considered perfectly normal for most reproductive tissues, somewhat paradoxically, this "ON at puberty – OFF at menopause" is not considered the same way for the skeleton. The prevailing pathomechanistic views consider the female skeletal mass that exists before menopause "normal", and accordingly, the loss of mineral occurring at menopause "pathological".

When Fuller Albright first described the disease called postmenopausal osteoporosis in 1940, he proposed the existence of estrogen-induced deposition of "reproductive bone" (medullary bone)[3]. Although this finding of the pubertal effects of estrogen on female bones was paramount to Albright when he devised his theory, it was not until 1998, that the existence of this phenomenon was truly "discovered" in humans[4]. By reanalyzing the data of whole body bone mineral content (TBMC) and body composition in Argentine boys and girls from 2 to 20 years of age[5], Schiessl[4] was able to show that the increase in bone mass in both sexes seems to closely follow the increase in lean body (muscle) mass until just prior to menarche, i.e., the onset of cyclic estrogen secretion. Thereafter, this uniform pattern in the development of male and female skeletons suddenly dissociated, as the female skeletal mass starts to increase rapidly and disproportionately to the concurrent increase in lean body mass[4]. This suggested that relative to the mechanical demands placed on bones, girls have substantially higher BMD than boys at the corresponding age. Gender studies carried out in female rats showed that the 'extra' bone laid down at menarche was deposited in the trabecular compartment, while male rats optimized their bone structure mostly in cortical bone improving its geometry.

If estrogen indeed is responsible for deposition of extra stock of trabecular bone into the female skeleton at puberty, then shouldn't withdrawal of estrogen secretion at menopause result in unpacking of roughly the same amount of mineral? The data from a study by Ferretti[6], in which TBMC and lean body mass were measured in a population of both genders between 2-87 years of age showed indeed, that at puberty the BMC/lean body mass–ratio in females reaches a higher level than that of males, and remains higher throughout the entire fertile period. At menopause, the BMC/lean body mass-ratio in females begins to decline rapidly and eventually exhibits comparable values to males, thus providing a quite convincing corroborative clinical evidence for the above noted packing and subsequent unpacking of bone at puberty and at menopause, respectively[6].

So, what does this all mean? In females, the onset of estrogen secretion at puberty results in the deposition of a surplus of trabecular bone in the axial skeleton, an apparent evolutionary safety measure against the anticipated transient bone loss caused by late pregnancy and lactation. When the female reproductive capacity ceases at menopause, this bone stock is shed as redundant, serving as the origin of the type I postmenopausal osteoporosis[7,8]. The female skeleton has to serve a dual purpose to serve its mechanical and reproductive functions. In the post-reproductive phase in the female, cortical geometry can no longer adapt sufficiently to substitute for the loss in mechanical stability caused by the shedding of the 'reproductive' cancellous bone compartment in the axial skeleton.

REFERENCES:

[1]Compston JE 2001. Physiol Rev 81:419-47. [2]Riggs et al 2002. Endocr Rev 23:279-302. [3]Albright F et al 1941. JAMA 116:2465-74. [4]Schiessl H et al 1998. Bone 22:1-6. [5]Zanchetta JR et al 1995. Bone 16:393S-9S. [6]Ferretti JL et al 1998. Bone 22:683-90. [7]Jarvinen TL 2003. J Musculoskelet Neuronal Interact 3:374-80. [8]Jarvinen TL et al 2003. J Bone Miner Res 18:1921-31.

Preparation of a Resorbable Osteoinductive Tricalcium Phosphate Ceramic

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Introduction

Over the past decade we have demonstrated numerous times that calcium phosphates can be rendered with osteoinductive properties by introducing specific surface microstructures[1]. Since most of these calcium phosphates contained hydroxyapatite, they are either slowly or not resorbable[2]. Resorbability is an often sought after characteristic of calcium phosphates so that they can be gradually replaced by newly formed bone. The objective of this study was to prepare a resorbable surface microstructured tricalcium phosphate (TCP) ceramic and evaluate its osteoinductive property and resorption rate after intramuscular implantation in dogs. This material was then compared to the established and slowly resorbable osteoinductive biphasic calcium phosphate ceramic (BCP).

Materials and Methods

Calcium phosphate ceramics: Calcium phosphate powders with the Ca/P ratios of 1.61 (BCP) and 1.50 (TCP) were mixed with diluted H_2O_2 solution and naphthalene particles to produce slurries. After foaming, drying and evaporation of the naphthalene, the materials were sintered for 8 hrs at 1150°C (BCP) or 1100°C (TCP). After milling, ceramic particles with a size of 1 to 3mm were sieved, cleaned and steam sterilized.

Chemistry and microstructures: The materials of interest were evaluated by XRD, and their microstructures were observed with SEM.

Calcium release: 0.5 ml ceramic particles (n=3 per material) were soaked in 100 simulated physiological solution (SPS) at pH 7.3 and 37±1°C for 200 minutes, the calcium concentration was measured continuously with a calcium electrode.

In vivo study: 1.0 ml ceramic particles (600±10mg per material per implant) were implanted in the paraspinal muscles of dogs (n=21). The animals were sacrificed after 6 weeks (8 animals), 12 weeks (8 animals) and 52 weeks (5 animals) respectively.

***Histology and histomorphometry*:** The samples harvested were fixed, dehydrated and embedded in MMA. Non-decalcified sections were made cross the middle for histological observation. The area percentage of bone and materials was measured by histomorphometrical analysis. Paired t-test was performed to evaluate the difference and $p<0.05$ was set as the significant difference.

Results

BCP was composed of 20±5% β-TCP and 80±5% HA by weight as shown in XRD analysis, while TCP was comprised of more than 90% β-TCP phase. Similar microstructures (grain size and micropores) were observed under SEM for the BCP and TCP ceramics. TCP showed a higher calcium release than BCP in SPS7.3.

Table 1. A summary of bone and materials in implants at different time points.

		6wks	12wks	52wks
BCP	Bone incidence	7/8	8/8	5/5
	Bone%	1±1	11±6	15±5
	BCP	53±4	44±8	43±4
TCP	Bone incidence	2/8	8/8	5/5
	Bone%	0.02±0.02	15±10	26±6
	TCP%	53±4	33±4	13±3

The tissue responses to BCP and TCP ceramic particles, with regard to bone formation and material resorption are summarized in Table 1 and illustrated in Figure 2, right. For both TCP and BCP, the area percentage of bone increased from 6 weeks to 12 weeks ($p<0.05$), while there was no significant increase after 12 weeks. Difference in bone formation between BCP and TCP was not seen although it should be mentioned that the absolute area percentage of bone was smaller in time for TCP due to degradation of this material.

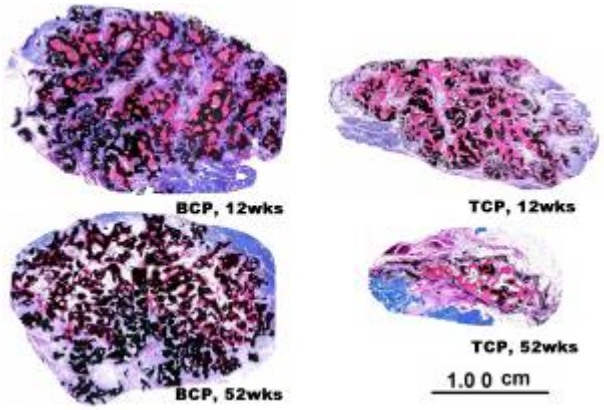

Figure 1. Histological overview of BCP and TCP implanted in muscle of dogs for 12 weeks and 52 weeks showing bone formation and resorption of TCP with time as compared to BCP.

Resorption of BCP was hardly seen either histologically (Figure 1) and histomorphometrically (Figure 2, right), while resorption of TCP from 6 weeks to 52 weeks was evident ($p<0.05$), as indicated by the decrease of implant size (Figure 1) and the signs of cell-mediated resorption (Figure 2, left). More than two third (2/3) TCP was resorbed after 52 weeks of implantation.

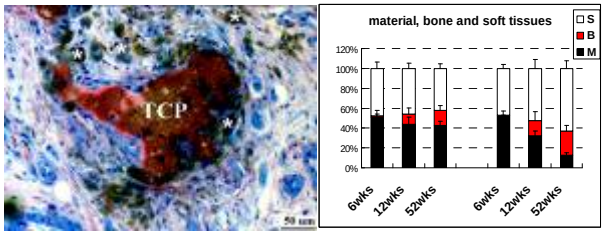

Figure 2. Left, cell-mediated resorption of TCP at week 12; right, percentage of bone, materials and soft tissues in BCP and TCP implants at different time periods.

Discussion and conclusion

We have successfully developed a surface microstructured, degradable tricalcium phosphate that has excellent osteoinductive properties. The osteoinductive potential was similar between TCP and BCP. Resorption of the TCP in time was demonstrated both histologically and histomorphometrically. Both chemical dissolution and cell-mediated resorption was observed resulting in almost 90% TCP loss after 1 year of implantation.

References

1. Yuan H, de Groot K. Calcium phosphate biomaterials: An overview. In Reis R.L., Weiner S., Learning from Nature How to Design New Implantable Biomaterials, Netherlands, Kluwer Academic, 2004, 37-57.
2. Yuan H, Yang Z, De Bruij JD, De Groot K, Zhang X. Material-dependent bone induction by calcium phosphate ceramics: a 2.5-year study in dog. Biomaterials, 22, 2617, 2001.

Standardized Augmentation of Osteoporotic Bone for Improved Implant Performance

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INTRODUCTION: The number of fragility fractures due to osteoporosis will strongly increase in the next decades. Implant fixation in the porotic bones of mainly elderly patients is difficult due to the limited purchase given in the weak trabecular network. In the AO Development Institute, we have conducted a number of studies to enhance implant purchase in the osteoporotic bone via augmentation with poly(methylmethacrylate) (PMMA) bone cements. A summary of the studies and their results is presented in this talk.

STUDIES AND OUTCOMES: Irrigation of the trabecular bone structure for fat removal was done with 200 to 500 cc of Ringer's solution. This led to significantly better control of cement distribution around a perforated implant (Fig 1.).

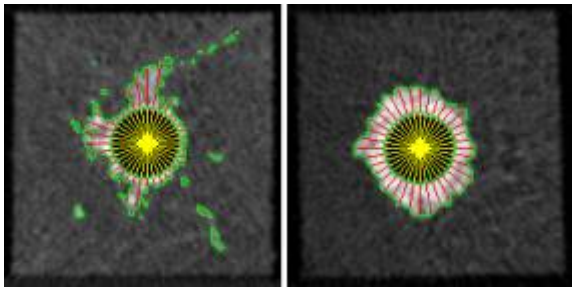

Fig. 1: Left: cement distribution after injection through a perforated implant (green pixels indicate perimeter of cement). Right: the same procedure led to significantly better control of cement distribution after irrigation and fat removal in cancellous bone.

The biomechanical studies of bone augmentation included cyclic testing of human femoral heads instrumented with DHS screws, with and without PMMA augmentation after irrigation in a left-right comparison. Cox regression analysis showed a significantly better performance of the augmented femoral heads, especially at low bone mineral density (Fig. 2 and 3).

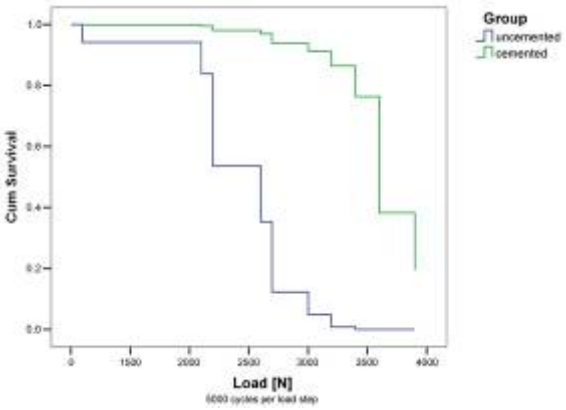

Fig. 2: Survival analysis of cemented and conventionally fixed DHS in femoral heads. There is a significant increase in performance, especially around physiologic loads.

Fig. 3: Typical appearance of a cut-out after mechanical testing. The augmented specimen went through the entire testing protocol (3.0 cc of PMMA cement around screw).

Temperature investigations around implants and setting PMMA cement (3.0 vs. 6.0 cc of cement) revealed the harmlessness of augmentation around a metallic implant.

Table 1: Average max. temperatures measured in different distances from the implant.

	3.0cc PMMA	6.0cc PMMA
PMMA	42.2 °C	45.6 °C
Interface	40.1 °C	41.7 °C
Close zone	39.1 °C	40.5 °C
Far zone	38.3 °C	39.8 °C

CONCLUSION: The newly developed standardized augmentation technique for fixation of osteoporotic proximal femur fractures seems to be promising to avoid implant cut-out.

A novel sheep model for evaluating biomaterials in cancellous bone

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INTRODUCTION: The use of sheep cancellous bone models is now well established for the assessment of new orthopaedic biomaterials and implants during either in vivo biocompatibility or corrosion studies. However, sheep have a limited availability of cancellous bone for implantation of biomaterials or surgical implants making it difficult to find multiple comparable sites within a same animal. Currently, one recommendation is to use the proximal and distal humerus and the proximal and distal femur for the implantation of a maximum of 8 different sites. These sites have different amounts of overlying soft tissue, and loading pattern which may effect the evaluation of implants.

The objective of this study was to develop a novel sheep model in which multiple implants can be tested in cancellous bone within the same animal. It was hypothesized 1)that the ovine distal femur and proximal tibia contain enough cancellous bone to allow multiple implants testing within the same animal and 2)that in vivo multiple implants application in the distal femur and proximal tibia within the same sheep is associated with minimal complications.

Materials & Methods

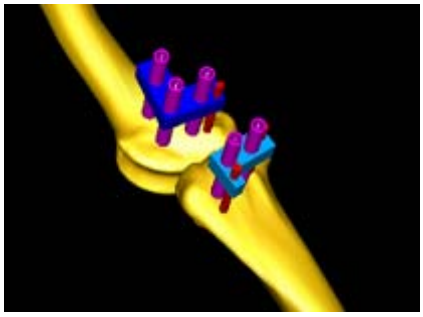
Cadaver studies: Studies were performed to both characterize ex vivo the cancellous bone tissue contained in the ovine distal femur and the proximal tibia and to develop ex vivo new surgical instrumentation for multiple implants application in the ovine distal femur and proximal tibia cancellous bone.

In vivo studies: Implants - Cylinders (5mm x 15mm) of bioresorbable polymer-ceramic composite, based on poly (L-lactic acid) (PLA) and β-tricalcium phosphate (β-TCP) were implanted in the distal femur and proximal tibia during a bioperformance in vivo study and 316L stainless steel cannulated screws in combination with guide wires of different material were implanted during an in vivo corrosion study. Animals - Mature, female, Swiss alpine sheep were obtained from a flock maintained for orthopaedic research, such that size, shape and age were standardized, and the health status known.

Surgical technique for the in vivo bioperformance study: Operated animals were placed under general anesthesia in lateral recumbency with the medial aspect of the most dependent hind limb clipped and prepared for aseptic surgery. The collateral ligament of the medial femoro-tibial joint was identified and two 20G, 1 inch hypodermic needles were placed in the femoro-tibial joint on both side of the collateral ligament. A 10-cm incision was made over this ligament, extending through the medial femoral fascia and splitting the distal head of the gracilis muscle. Three-hole and 2-hole jigs were positioned; 1 cm from the joint space, on the distal femur and the proximal tibia respectively after surgical exposure was obtained (Fig1). A 5.1 mm depth-regulating drill bit was used to drill five 15 mm deep holes in the cancellous bone. The tested bioresorbable polymer-ceramic composite implants were inserted in these holes. Positional screws were placed to guide a jig made for harvesting implants following euthanasia. The surgical incisions were closed routinely in 3-layers. The sheep were changed to the opposite lateral recumbency and the second hind limb was operated in a similar way.

Surgical technique for the in vivo corrosion study: Positioning, preparation and surgical exposure was performed as described above. A K-wire was inserted in the middle of the medial femoral condyle and the proximal tibia, cranial to the medial collateral ligament and 1 cm distal to the joint space. Their placement was verified with fluoroscopy. Three-hole and 2-hole jigs were slid over the K-wires (Figure 1). Stab incisions were made and bone holes drilled to insert the tested implants. The wound was closed in two layers. The sheep were changed to the opposite lateral recumbency and the second hind limb was operated in a similar way.

Analgesia protocol and postoperative management: Systemic and local analgesia consisted of nerve blocks and pre-, intra- and postoperative administration of NSAIDs and opioids. The sheep were maintained in single pens until shortly after suture removal to avoid damage to the incision, and were then group housed.



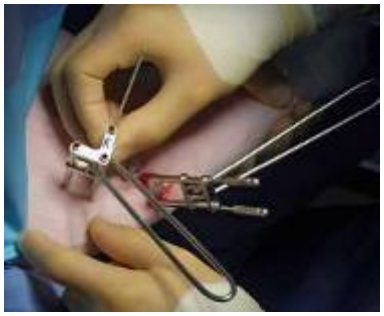


Figure 1. Jigs positioning for the biocompatibility study (above) and corrosion study (below).

Results & Discussion: Twelve sheep were included in the biocompatibility study and 18 in the corrosion study. No postoperative complications were recorded in 28 sheep. Two sheep were euthanized within 2 weeks of surgery: one with unresponsive pleuropneumonia and one with septic femoro-tibial arthritis. The described techniques allowed the insertion of 10 implants per animal. The designed jigs allowed accurate and reliable placement of all the implants in cancellous bone (Fig 2).

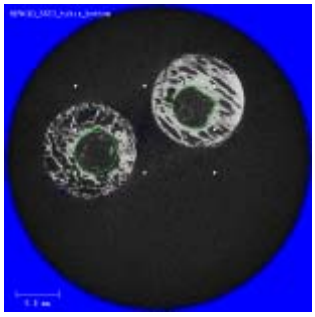


Figure 2. Radiographs confirming placement of the implants in cancellous bone, the harvested core and CT images of the cores.

Conclusion: This abstract describes a useful, low-morbidity, animal model for testing multiple biomaterials in cancellous bone in the same animal. Differences exist in the trabecular structure of the proximal tibia compared with the distal femur which should be considered in the experimental design. The size of implants tested is also limited. However the condensed spatial assignment of samples ensures shorter surgery times, the opportunity for effective local anaesthesia, and more similar loading patterns and amounts of overlying soft tissue compared with other models. Care is required to ensure that implants located in the distal most position of the femur do not penetrate the intercondylar notch. Images taken in two orthogonal planes are recommended. The technique also allows accurate harvest of implants, performed by coring out a bone plug surrounding the implant for detailed imaging and histological analysis (Figure 2).

Influence of the Mechanical Environment upon the Healing of Segmental Bone Defects in a Rat Model Studied with a Novel External fixator

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INTRODUCTION: Despite the intrinsic ability of bone to heal, there are still numerous clinical circumstances where bone healing is defective and demands the attention of the physician. Common examples include the delayed and non-union of fractures, and the loss of large segments of bone after traumatic injury, tumor resection and failed arthroplasty. There are several surgical approaches to enhance the healing of human bones, and these have been supplemented in recent years by the introduction of recombinant, human bone morphogenetic proteins -2 and -7 (BMP-2 and BMP-7) into clinical practice in combination with different fixation devices. Over the years there have been many advances in technology, which helped to improve the fixation for critical sized defects. Despite these improvements, the clinical management of critical-sized defects remains problematic for surgeons today because it isn't clear how stiff or flexible the fixation device should be to optimize bone healing. Our project is based on the hypothesis that the healing of critical-sized defects in response to BMP-2 can be dramatically improved by manipulating the mechanical environment within the defect. Accordingly, this project aims to investigate the influence of the mechanical environment on bone healing in response to BMP-2 in a rat, critical-sized defect model. The mechanical environment of the defect is altered with a custom made external fixator whose stiffness can be changed in a reliable, quantitative fashion.

METHODS: Novel, second generation, external fixators that can be adjusted to provide three different stiffnesses were designed specifically for the rat femur. All materials in the fixator were chosen to be the same as used clinically for human implants. The screws for a new external fixator are made out of titanium (Ti). The stability bars are made out of polyetheretherketones (PEEK). The diameter of the screws is 1mm and the length is 12.75mm. The distance between the screws is 4mm and the distance between the middle screws is 11mm. All holes are predrilled using a 0.79mm drill bit. The screws are locked in the corresponding holes of the fixator, which is parallel to bone surface and set at the distance of 5mm. The fixator stiffness can be changed by changing the stability bars to provide stiffnesses of 40, 70 or 100%, as required. To create reproducible 5mm defects in all rats the guide was designed for a giggly saw that clips on the external fixator in between the two middle screws. A rat, femoral, critical-sized defect model was used to test this fixator in-vivo. A 5mm defect was created in 6 Sprague-Dawley rats and treated with rhBMP-2 applied on a collagen sponge with external fixators providing 40, 70, 100% stiffness. Animals were x-rayed weekly for 8 weeks to monitor bone healing.

RESULTS*:* A second generation external fixator prototype was successfully created and manufactured for testing in-vivo (Figure 1). An in-vivo pilot study showed that the there is a difference in bone healing depending on external fixator stiffness. Weekly x-rays revealed that bone callus size was biggest in the group with the lowest stiffness (40%) fixator. Furthermore, early callus formation was seen in this group and 70% stiffness group after 9 days of treatment. However, the group with 100% stiffness external fixator had no callus formation after 9 days of treatment. In this group callus formation was delayed until after two weeks of treatment. By the third week defects were bridged with all fixation methods with the biggest callus in 40 and 70% stiffness fixators and smaller callus with 100% stiffness (Figure 2).

Figure 1 External Fixator with saw guide

DISCUSSION & CONCLUSIONS: Loss of large segments of bone leads to critical-sized defects that fail to heal spontaneously. Although

healing can be induced by recombinant, human bone morphogenetic protein-2 (rhBMP-2), the clinical response is modest. The current project focuses on the influence of the mechanical environment on the healing of critical sized segmental defects in response to rhBMP-2. In preliminary studies, using a first generation external fixator to stabilize the defect, we have been able to achieve osseous union using rhBMP2 and BMP-2 gene transfer with an adenovirus vector. However the rate of healing and the mechanical properties of the healed bone are inconsistent. We hypothesized that this is due to insufficiencies in the rigid external fixator, which generates an unfavorable local mechanical environment. Therefore, we have designed a new, second generation external fixator which allows us to control and measure with precision, the mechanical environment within the critical-sized segmental defect in-vivo. Our pilot study shows that fixator stiffness is important in the biologic process of healing bone. In fact, from the weekly x-rays we observed that with 40 and 70% stiffness fixator callus formation was seen already after 9 days of treatment, whereas defects subjected to 100% stiffness had no callus. Furthermore, after two weeks bone regeneration was seen in all groups, but two lower stiffness groups had bigger callus formation as compared to the stiffest fixator. This confirms findings in the literature that if there is too much micro motion during healing, bone bigger callus forms (1) Material, structural and mechanical testing will be performed to determine how important early callus formation and size is to the physical properties of the healed bone. We will apply this knowledge to manipulate fixator stiffness through the course of healing to get rapid bone regeneration and best quality of healed bone. The findings from this study will be highly relevant to the surgical management of patients with segmental bone defects and, possibly, delayed and non-union fractures.

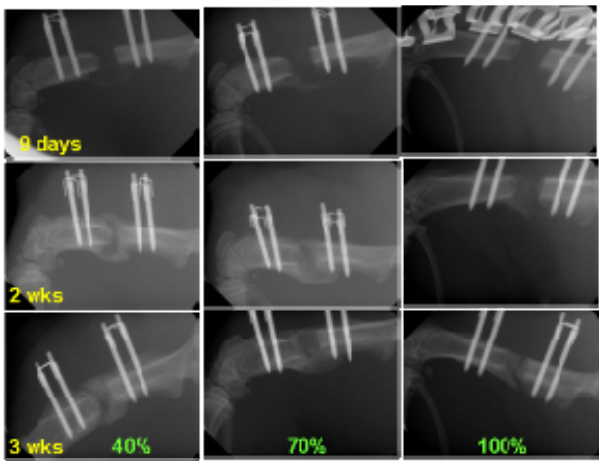

Figure 1 Representative x-ray images with 40, 70 and 100% stiffness external fixator after 9 days, 2 and 3 weeks of treatment with rhBMP-2.

REFERENCES: 1. Gomez-Benito MJ, Garcia-Aznar JM, Kuiper JH, Doblare M 2006 A 3D computational simulation of fracture callus formation: influence of the stiffness of the external fixator. J Biomech Eng 128(3):290-9.

Cell Therapy of Bone

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INTRODUCTION: We recently reported the 7 year follow-up repair of large bone defects in humans by autologous in vitro expanded bone marrow stromal cells (BMSC) seeded onto a 100% HA porous ceramic. Despite the success of the bone repair we failed to observe complete bone regeneration due the low resorbability of the porous HA bioceramics. In the attempt to improve the tissue engineering approach, we tested in vitro and in two animal models a new resorbable porous scaffold, based on silicon-stabilized tricalcium phosphate ceramic biomaterial.

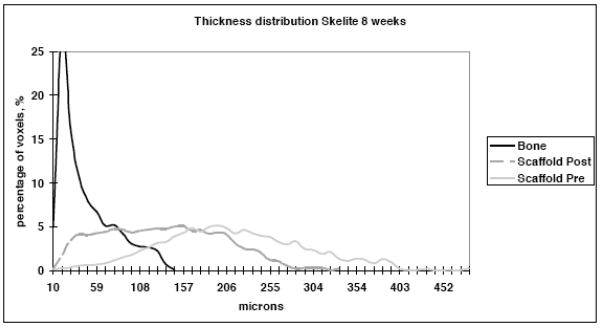
METHODS: *Sheep model***:** Critical size defects of the tibial mid diaphysis of sheep were treated with autologous bone (control group) or with the ceramic scaffold seeded or not with expanded BMSC. An internal locking plate was applied for stabilization. Animals were sacrificed at different times during the 10-months follow-up period. To determine bone and scaffold percentages, tissue morphology, and vascular density, radiography, microradiography and histology were performed.

RESULTS: In the autologous bone group new bone synthesis was already observed in the first follow-up radiographs, but stopped after 20-24 weeks from surgery reaching only 70-80% of the normal bone value. No bone formation occurred in implants not seeded with cells. A bone deposition occurred in the cell-seeded scaffolds starting from the periphery of the bone stumps where a higher vascular density was observed. This effect ended within 20-24 weeks, as for the autologous bone, suggesting similar kinetics of the repair processes involved. BMSC-loaded ceramics displayed a progressive scaffold resorption, coincident with new bone deposition.

To investigate the coupled mechanisms of bone formation and scaffold resorption, X-ray computed microtomography with synchrotron radiation (μCT) was performed on BMSC-seeded ceramic cubes before and after implantation in immunodeficient mice for 2 or 6 months. All scaffolds presented a uniform density before implantation. After being seeded with cells and their in vivo implantation, the *same scaffolds* presented a decreased scaffold trabecular thickness and areas of different segregated densities. A μX-ray diffraction analysis performed on sections of the same samples revealed that in the contact areas between the newly formed bone and the scaffold, the TCP component of the ceramic decreased much faster than the HA component. In scaffolds implanted without cells, both the ceramic density and the TCP:HA ratio remained unchanged with respect to the pre-implantation analysis

DISCUSSION & CONCLUSIONS: The, porous ceramic implants, based on silicon-stabilized tricalcium phosphate combines good osteoconductive properties with the capability to be progressively resorbed during the bone repair/regeneration process. The coupling between scaffold resorption and bone formation is new and it is at variance to the results with other bone biomaterials that are either minimally resorbed in vivo, as hydroxyapatite, or more soluble one, as beta-TCP or bioglasses that may dissolve in vivo before bone formation and a proper tissue repair.

REFERENCES: [1]Quarto R, Mastrogiacomo M, Cancedda R, Kutepov S, Mukhachev V, Lavroukov A, Kon E, Marcacci M. New Engl J Med 344,385, 2001; [2]Marcacci M, Kon E, Moukhachev V, Lavroukov A, Kutepov S, Quarto R, Mastrogiacomo M and Cancedda R.Tissue Engineerig 13:947-955, 2007; [3]Mastrogiacomo M, Papadimitropoulos A, Cedola A, Peyrin F, Giannoni P, Pearce S.G., Alini M, Giannini C, Guagliardi A, and Cancedda R. Biomaterials 28,1376, 2007; [4]Papadimitropoulos A, Mastrogiacomo M, Peyrin F, Molinari E, Komlev VS, Rustichelli F and Cancedda R. Biotechnol Bioeng. 98,271, 2007



Characterization and Utilization of Mesenchymal Progenitor Cells Recovered with the Reamer-Irrigator-Aspirator

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INTRODUCTION: Trauma surgeons frequently need to repair large and difficult osseous defects. Central to this endeavour is a source of bone or bone precursors. The Reamer-Irrigator-Aspirator (RIA) is a new device that might supply these in a convenient and relatively non-invasive fashion. The RIA is inserted into the intermedullary canal and the "reamings" are harvested from an irrigating solution used in conjunction with the device. The reamings comprise osseous particles that are captured with a course filter, and "flow-through" that passes through the filter. While the osseous particles have been used to augment fracture repair, the "flow-through" has not been well characterized. Here we describe the isolation and characterization of mesenchymal progenitor cells recovered from reamings, and suggest ways in which they might be used to develop novel, intra-operative technologies for the efficient restoration of bone and other musculoskeletal tissues.

METHODS: Subjects (n=7) were 71-97 years of age, undergoing hip hemiarthroplasty. Osseous particles were collected by filtration and cells recovered by centrifugation, with or without Ficoll-gradient separation. Supernatants were assayed for BMP-2, IGF-1, FGF-2 and TGF-β_1 by ELISA. With certain reamings, osseous particles were placed into culture to allow the outgrowth of cells which were then harvested by trypsinising, and sub-cultured. The surface phenotype of the cells was analysed by FACS. Chondrogenesis, adipogenesis and osteogenesis were assessed using standard assays.

RESULTS: BMP-2 could not be detected in the reamings, but IGF-1 (4.9 $\pm$ 3.7 ng/ml), FGF-2 (430 $\pm$ 160 pg/ml) and TGF-β_1 (9.8 $\pm$ 5.9 ng/ml) were present. However, because of the diluting effect of the irrigant, these concentrations are relative. Filtrate cells were CD44+, CD90+, CD105+ and CD106+, but CD34- and CD45-, These cells were able to differentiate along adipogenic, chondrogenic and osteogenic lineages. Cells emerging from the osseous particles were indistinguishable from those recovered from the flow-through in terms of their morphology and ability to undergo multi-lineage differentiation. [1] The rich harvest of osteogenic cells was remarkable, given the age of these patients. The impressive osteogenic potential of the reamings encourages us to develop methods that will allow the trauma surgeon using the RIA to isolate osteoprogenitors, commit them irreversibly to osteogenesis, and re-implant them within a single operation. Thus we are defining conditions that, within 2 hours, will commit mesenchymal progenitor cells irreversibly to osteogenesis. These include transfer of osteogenic genes, exposure to proteosome inhibitors and histone de-acetylase inhibitors, among others. As noted first by Leboy's group [2], human mesenchymal progenitors do not undergo osteogenesis in response to rhBMP-2, but they respond well to dexamethasone, BMP-6 and BMP-9. Preliminary data suggest that cells recovered from the reamings are readily transduced in suspension by adenovirus vectors.

DISCUSSION & CONCLUSIONS: The RIA provides the trauma surgeon with new possibilities for greatly enhancing bone healing in a convenient and affordable manner. The challenge is to define powerful osteogenic stimuli that will be effective within the short, intra-operative period.

REFERENCES: [1] R. M. Porter, et al (2008) *J Orthop Res* Submitted.. [2] D. L.Defenderfer, et al (2003) *J Bone Jt Surg* 85A: 19-28.

ACKNOWLEDGEMENTS: This study was supported by the AO Research Fund (project no. 04-B86) of the AO Foundation (Davos, Switzerland). RMP was supported by a postdoctoral Ruth L. Kirschstein National Research Service Award (F32 EB005566) from the National Institute of Biomedical Imaging and Bioengineering (Bethesda, MD). AI is supported by the Fulbright Foundation. Synthes donated the RIA system and accessories.

The many roles of the extracellular calcium-sensing receptor, CaR, in osteoblast biology

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INTRODUCTION: Fluctuations in extracellular free ionized calcium concentration ($[Ca^{2+}]_o$) occur naturally during bone remodeling and contribute to systemic Ca^{2+}_o homeostasis [1]. In tissue culture models, elevation in $[Ca^{2+}]_o$ induces osteoblast chemotaxis and proliferation [2,3] and alter the levels of expression of several osteoblast differentiation markers [4,5]. Whether these effects are mediated by a functional calcium-sensing receptor, CaR [6] expressed in bone is controversial.

METHODS: Immunohistochemistry was performed to detect CaR expression in freshly frozen, undecalcified preparations of human mandible and rat femur. In addition, we have used primary and established models of osteoblasts, fetal rat calvarial (FRC) and the murine clonal cell line, 2T3 cells, to investigate the expression of the CaR and to study the effects of known CaR agonists (Ca^{2+}, Gd^{3+} and the known anti-osteoporotic agent strontium, Sr^{2+}) on acute, mitogenic outcomes (ERK1/2 phosphorylation), and chronic, differentiation-dependent cellular responses (expression of osteoblast differentiation markers, core binding factor α1 [cbfa1] and osteopontin, and mineralization). The ability of the negative allosteric modulator (NPS 89636) to affect such responses was also investigated.

RESULTS: CaR mRNA and protein were detected in both human and rat osteoblasts and osteocytes and in primary and established models of osteoblasts, FRC and 2T3 cells. Elevating $[Ca^{2+}]_o$ and treatment with non-permeant CaR agonists, Gd^{3+} and Sr^{2+}, resulted in activation of pro-proliferation and pro-survival signals. Expression of the osteoblast differentiation markers cbfa1, osteocalcin, osteopontin and collagen I mRNA and/or protein were increased by high $[Ca^{2+}]_o$ and/or by treatment with Sr^{2+}, as was mineralized nodule formation. The calcilytic 89636 prevented Ca^{2+}_o-, Gd^{3+}- and Sr^{2+}-dependent responses in both FRC and 2T3 cells.

DISCUSSION & CONCLUSIONS: Small deviations of $[Ca^{2+}]_o$ from physiological values directly and profoundly affect osteoblast function, through the CaR and independently of systemic calciotropic hormones. In addition, the beneficial effects of strontium ranelate as an anti-osteoporotic agent can be ascribed, at least in part, through stimulation of the osteoblast CaR. Pharmacological modulators of CaR function are currently available on the market for the treatment of hyperparathyroidism secondary to kindey failure. The possibility to use positive and negative allosteric modulators of the CaR for bone-related disease is currently being explored.

REFERENCES: [1] Parfitt, A. M. (1987) *Bone* 8 Suppl 1, S1-8; [2]Godwin, S. L. & Soltoff, S. P. (1997) *J Biol Chem* 272, 11307-12; [3]Yamaguchi, T., Chattopadhyay, N., Kifor, O., Butters, R. R., Jr., Sugimoto, T. & Brown, E. M. (1998) *J Bone Miner Res* 13, 1530-8; [4]Eklou-Kalonji, E., Denis, I., Lieberherr, M. & Pointillart, A. (1998) *Cell Tissue Res* 292, 163-71; [5]Nakade, O., Takahashi, K., akuma, T., Aoki, T. & Kaku, T. (2001) *J Bone Miner Metab* 19, 13-9; [6]Brown, E., M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M., A., Lytton, J. & Hebert, S., C. (1993) *Nature* 366, 575-580.

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Improving the Osteogenic Behavior of Human Mesenchymal Stromal Cells

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INTRODUCTION: Bone marrow contains mesenchymal stromal cells (MSCs), which can undergo osteoblastic differentiation when cultured under the appropriate conditions. The utilization of MSCs to generate osteoblasts presents an attractive opportunity for addressing orthopaedic problems that require substantial bone formation. To achieve this goal, it is first necessary to develop protocols that optimize MSC osteogenesis. Recombinant BMP-2 and BMP-7 are in clinical use for bone healing, but MSCs of human origin are only moderately responsive to these proteins, and deposit limited amounts of mineral *in vitro* in response to them (1). Recent reports indicate that other BMPs may be more important for hMSC osteogenesis (2). Another promising strategy is pre-treatment of these cells with histone deacetylase inhibitors (HDIs) (3). Here, we investigated the effects of BMP-6, BMP-9and HDIs on the osteogenic behavior of hMSCs in monolayer culture.

METHODS: Human bone marrow was obtained from patients undergoing primary hip arthroplasty, and the mononuclear cell fraction was cultured on tissue culture plastic in order to isolate and expand the number of MSCs. Second-passage cells were plated at a density of 10^4 cells/well in 24-well plates containing basal medium (low-glucose DMEM with 10% fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 50 µg/ml ascorbic acid, and 10 mM β-glycerol phosphate). After 24h, rhBMP-2 (100 ng/ml), rhBMP-6 (100 ng/ml) or rhBMP-9 (100 ng/ml) were added to the cultures..Additional hMSCs were cultured under similar conditions to test the effects of pre-treatment with two clinically-used HDIs: valproic acid (VPA) and trychostatin A (TSA). After 24h in basal medium, hMSCs were pretreated for 4 days with increasing concentrations of VPA (1, 3 or 5 mM) and TSA (100, 500 or 1000 nM). After 4 days osteoblastic differentiation was induced by supplementing the medium with dexamethasone (10^{-7}M); untreated hMSCs were used as controls. Alkaline phosphatase (ALP) activity (normalized by DNA content) was analyzed at day 10 as a marker of early osteoblastic differentiation. Alizarin red staining and calcium assay were performed at day 21 as indicators of mineral deposition.

RESULTS: Treatment of monolayer cultures of hMSCs with rhBMP-6 and -9 resulted in moderately better mineral deposition than treatment with rhBMP-2 (*Figure 1*). Furthermore, pre-treatment with VPA or TSA prior to the initiation of differentiation also increased mineral deposition in a dose-dependent manner.

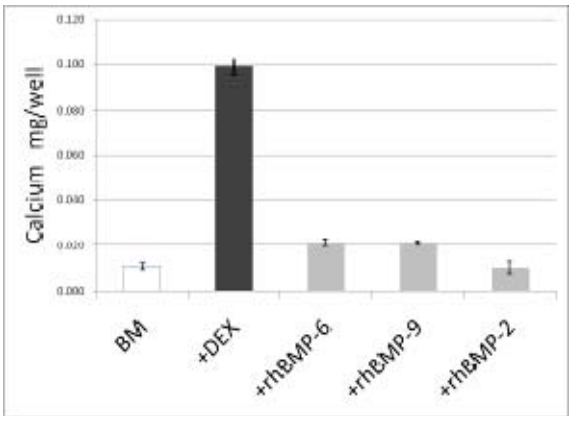

Fig. 1: Calcium deposition in response to rhBMP-6, -9, -2.

DISCUSSION & CONCLUSIONS: The data suggest that BMPs -2 does not effectively induce osteogenesis in isolated hMSCs cultured as monolayers. BMP-6 and –9 moderately enhance the osteogenic differentiation of hMSCs but they are far less potent than dexamethasone in this regard. These findings are intruiging, given the popularity of using recombinant BMPs in the clinic. Pre-treatment of hMSCs with clinically available HDIs (VPA and TSA) also enhanced osteogenic differentiation, which implies that HDIs provide an additional strategy for enhancing bone engineering.

REFERENCES: [1] D.L. Diefenderfer, A.M. Osyczka, J.P. Garino, et al (2003) *J Bone J Surg* **85**:19-28. [2] M.S. Friedman, M.W. Long, and K.D. Hankeson (2006) *J Cell Biochem* **98**:538-54. [3] H.H. Cho, H.T. Park, Y.J. Kim, et al (2005) *J Cell Biochem* **96**:533-42.

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Control of osteoblast genotype with implant surface microtopography.

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INTRODUCTION: Complications relating to excessive bony overgrowth of an internal fixation device account for approximately 13% of all removal-related morbidity problems. We have previously shown *in vivo*, that surface polishing can significantly reduce the percentage of bone contact to an implant[1,2]. Consequently, a lower removal force is required for extraction[1,]. However, we do not believe this effect to be exclusively mechanical. Specifically, we hypothesis that both material type and surface micro-topography will influence the temporal expression of genes 'specific' to an osteoblast genotype, as well as reducing expression of factors essential for differentiation, matrix production and mineralisation, and therefore, bone formation. Thus, to elucidate the effect of surface polishing on bone over-growth from a cellular view point, here we have investigated *in vitro*, alterations in osteoblast genotypic expression due to surface polishing.

METHODS: 50mm diameter samples were fabricated from 'standard' orthopaedic grade commercially pure titanium (cpTi), titanium6%-aluminium-7%niobium (TAN) & titanium-15% molybdenum (Ti15Mo) (positive controls for osseointegration). Experimental surfaces were prepared via electro- & paste polishing the above materials. Stainless steel was included as a negative control. Surface characterisation involved SEM, AFM, XPS, contact angle & non-contact profilometry. Cellular response was assessed using primary rat calvarial (RC; 100,000 cells/sample) cells pertaining to the relative fold change in mRNA (osteocalcin (OCN), Alkaline phosphatise (ALP), bone sialoprotein (BSP), Cbfa1, collagen I and osterix) after 7, 14 and 21 days culturing.

RESULTS: No significant difference was observed for Cbfa-1 mRNA levels on TE (p=1.000) or TP (p=1.000) compared to TS, nor for NE (p=1.000) or NP (1.000) compared to NS, nor ME (p=1.000) or MP (0.619) compared to MS. Osx up-regulation on TS was significantly different to Osx up-regulation on TP (p=0.48), but not TE (p=0.086). No significant differences for osx regulation were observed for NE (1.000) or NP (p=0.794) compared to NS. No significant differences were reported for osx expression on ME (p=1.000) or MP (p=1.000) compared to MS. ALP expression profiles for TE (p=1.000) and TP (p=1.000), did not significantly differ from those accrued for TS or for NE (p=0.257) or NP (p=1.000) compared to NS. ALP expression for ME (p=1.000) and MP (p=0.506) samples was not found to be significantly different to that observed for MS. No significant difference in COL1 regulation was observed for TE (p=1.000) or TP (p=0.672) samples compared to TS. A significant difference in COL1 expression was observed for NE (p=0.000) and NP (p=0.000) compared to NS samples. Cells cultured on MP had a significantly lower COL1 expression profile compared to ME (p=0.000), and MS (p=0.018). No significant difference was observed for BSP expression on TE (p=1.000), or TP (p=1.000) compared to TS, or for NE (p=0.465) or NP (p=0.556) compared to NS samples, or for ME (p=1.000) or MP(p=1.000) compared to MS. OCN expression for TS was noted to be significantly higher compared to TE (p=0.001) and TP (p=0.000) samples. No significant difference in OCN regulation was observed for cells cultured on NS samples compared to NE (p=0.419), NP (p=0488). However, MS samples compared to ME (p=0.005), MP (p=0.049) showed significant difference in OCN expression.

DISCUSSION: Previous *in vivo*[1,2] studies from our laboratory have highlighted the promise of surface polishing for ease of removal of temporary fixation devices. This influence will inadvertently improve surgical related complications associated with removal, as well as reducing the economic costs associated with removal related morbidity. To help elucidate the cellular regulatory mechanisms of this influence, in this study, we have investigated *in vitro* the influence of surface polishing on osteoblast genotype, as we believe the effect of polishing not to be purely mechanical. As hypothesised, both material type and surface polishing significantly affected osteoblast genotype. In this study, compared to standard samples, generally, no significant difference was observed for Cbfa-1, osx, ALP, COL1 or BSP expression compared to standard counterparts. Thus it is reasonable to speculate

that from the initial signalling of Cbfa-1, cascades involved down stream of this factor to the point of initial crystal nucleation and apatite growth, remain engaged for cells cultured on polished samples. The exception to this was polished TAN samples, which had significantly lower COL1 mRNA levels, but no difference in OCN expression. The observation in this study that BSP expression is similar for both polished and standard micro-rough samples, but OCN is significantly decreased on polished samples is very interesting. This may be due to the relatively high concentration of BSP, or altered secondary structure on polished samples, which can inhibit apatite formation[3]. If this were the case, perhaps more time would be required for polished samples for sufficient apatite nucleation and crystal growth to occur, thus OCN expression would be subsequently reduced and/or delayed to allow for this phase of mineralisation to occur. Alternatively, BSP induction may be sufficient polished samples to induce initial phases of mineralisation but at a slower rate compared to standard samples, thus through an alternative signalling mechanism independent of Cbfa-1, or through Cbfa-1 binding with a co-factor specific for OCN repression[4], OCN is decreased, therefore, terminal differentiation would occur at a later time on polished samples. Since decreasing OCN allows mineralisation to occur, these results, regardless of the events involved in their occurrence, would suggest that terminal differentiation occurs at delayed rate on polished samples compared to micro-rough counterparts. This is an extremely poignant point, as this may elucidate the mechanism by which surface polishing effectively reduces bone over-growth compared to standard devices of similar material.

CONCLUSIONS: These findings indicate that the influence of surface polishing for reduced bone over-growth is in part attributable to alterations in genotype on a cell level. Ongoing studies are now focusing on alterations in cytoskeletal organisation and cell shape on polished surfaces as a potential cause for these distinct genotypic profiles.

REFERENCES:

1. Pearce et al., 2008. J. Orthop Res. (in press)
2. Vos *et al.*, 2008. JBJS, (submitted)
3. Stubbs JT et al., 1997 J Bone Miner Res.
4. Lamour et al., 2007 J Biol. Chem.

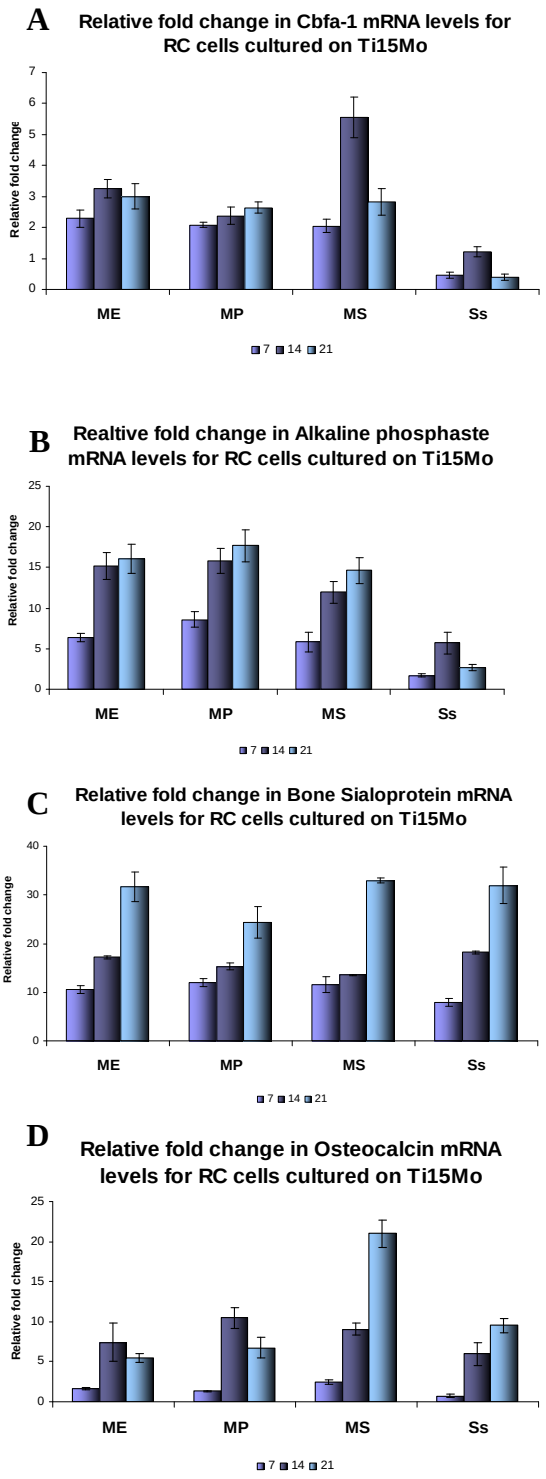

Fig. 1. Representative results for changes in mRNA levels of (A)Cbfa1, (B) ALP, (C) BSP and (D) OCN mRNA levels for RC cells cultured on electropolished (ME), paste polished (MP), and standard Ti15Mo (MS), with stainless steel (SS) as a control.

ACKNOWLEDGEMENTS:
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New insights into regeneration of intervertebral disc and spinal cord

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INTRODUCTION: The intervertebral disc(IVD) and the spinal cord are the two major components of the spinal column. Various medical problems such as degenerative disease to high-energy trauma induce alteration of function and organ structure. While the bone elements can be reconstructed using various bone grafts and tissue engineering techniques, biological regeneration of IVD and spinal cord has not yet been achieved. In this lecture, 2 studies will be presented assessing the possibility of utilizing intrinsic cells for regeneration of these organs.

METHODS:

Study 1. Can bone marrow mesenchymal cells be recruited for regeneration of IVD? IVD degeneration was applied to in mice whose BM had been replaced with BM cells from green fluorescent protein (GFP)-transgenic mice. Histological and immunohistochemical analyses were performed in 3 groups: Group D: degeneration, Group R: regeneration and Group C: control vehcle.

Study 2. Mobilization and recruitment of intrinsic cells from bone marrow for spinal cord regeneration. Spinal cord injury was applied at Th10 level by a static load(25g,5min) in mice whose BM had been replaced with BM cells from GFP-transgenic mice. Injured mice were separated into different 4 groups (Group A: Combination of SCF and G-CSF, Group B: SCF alone, Group C: G-CSF alone, Group D: PBS). G-CSF (300μg/kg/day) and SCF (100μg/kg/day) were subcutaneously injected in subacute phase (days 11 to 20). BrdU injections were performed to assess cell proliferation in peri-injured area. Neural markers were evaluated 4 weeks after injury and hind limb locomotor using BBB Scale was analyzed until 12 weeks after injury.

RESULTS&DISCUSSION:

Study 1. Histologically, severe degeneration was observed in Group D and moderate degeneration was observed in Group R. GFP+ cells were detected in end plate and annular/endplate junction in Group D and R, while no GFP+ cells were detected in Group C. GFP+ cells were also seen in nucleus pulposus in Groups D and R, but were few. Immunofluorescent analysis revealed that these GFP+ cells did not co-stain with hematopoietic markers; CD34, CD45 or Mac1, but co-stained with keratan sulfate. These findings suggest that induction of degeneration and sequential regeneration induces mesenchymal cells from the bone marrow to regenerate IVD structure. **Study 2.** No significant difference between groups was seen in GFP+ cells. The result showed that these cytokines were not effective for mobilization of BM-derived cells to injured spinal cord. Most of GFP+ cells were CD45+ and F4/80+ activated macrophage. Small portion of GFP+ cells with treatment were NG2+, indicating that they were oligodendrocyte progenitor cells (OPCs). However, differentiation of BM-derived cells to astrocyte and neuronal cells were not detected. The number of GFP- F4/80+ cells was significantly increased in Group A and most of them were negative for BrdU suggesting combined cytokine treatment was effective to recruit activated microglia to injured area from peri-injured area but not to proliferate the microglia in injured center. The number of GFP- NG2+ BrdU+ cells significantly increased in Group A which also demonstrated GFP- GSTy π + BrdU+ cells. These findings suggest that intrinsic OPCs were actively proliferating and some of them differentiated to mature oligodendrocyte. Locomotor of hind limb showed significant recovery after 6 weeks in combination group.

CONCLUSIONS:

Results of the current study demonstrate that intrinsic cells mobilize to the degenerated or injured site possibly to promote regeneration in IVD and spinal cord. These findings are informative in finding out the involvement of intrinsic stem cell niche in control of homeostasis of IVD and spinal cord. Modulation of these cells combined with growth factor treatment may be useful in IVD and spinal cord regeneration.

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Intradiscal Growth Factor Therapies for Intervertebral Disc Degeneration

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INTRODUCTION: Although the precise etiology of disc degeneration remains unknown, it may be surmised that the degenerative cascade can be slowed or halted by altering the homeostatic balance in favor of increased anabolic activity by intradiscal growth factor therapy. Moreover, growth factors may influence the inflammatory pathway, thereby having a direct effect on the pain associated with disc degeneration.

INTRADISCAL INJECTION OF GROWTH FACTORS PROMOTES REGENERATION: Thompson *et al.* first proposed to stimulate IVD repair by exogenous growth factors[1]. A number of studies have since shown that intradiscal injection of growth factors can be used to stimulate regenerative activity within the IVD. Walsh *et al.* compared the effects of single vs. multiple injections of growth and differentiation factor-5 (GDF-5), transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF) in a mouse-tail model of disc degeneration and demonstrated that intradiscal injection of either GDF-5 or TGF-β stimulated cell proliferation and extracellular matrix protein synthesis[2]. Subsequent studies in both juvenile[3-5] and adult[6] rabbits have shown that intradiscal injections of recombinant human osteogenic protein-1 (rhOP-1) or rhGDF-5 (Fig. 1) can stimulate regenerative changes in injury[3,4,6] and chemical[5] models of disc degeneration. *In vitro* studies have shown that in addition to anabolic stimulation, OP-1 can also down-regulate expression of matrix-degrading enzymes, such as ADAMTs-4[7] further tipping the homeostatic balance away from additional degeneration.

ANTI-INFLAMMATORY EFFECT OF GROWTH FACTORS: In addition to the effects of growth factors in controlling the balance of anabolic and catabolic activity, recent studies have shown that OP-1 can influence the levels of cytokines such as IL-1β, IL-6, and TNF-α both *in vitro*[7] and *in vivo*[8]. Moreover, *in vivo* pain assessment in rats has demonstrated that OP-1 reduced hyperalgesia in both disc degeneration[9] and herniation[10] models.

DISCUSSION: Since growth factor therapy requires cells with biosynthetic activity and a nutritional pathway adequate for supporting increased metabolic activity, it is expected that intradiscal growth factor therapy will only be effective for relatively early stage disc degeneration. Nonetheless, the promise of a minimally-invasive therapy for the treatment of degenerative disc disease is encouraging.

REFERENCES: [1]JP Thompson *et al.* (1991) *Spine* **16**:253-60. [2]AJLWalsh *et al.* (2004) *Spine* **29**:156-63. [3]K Masuda *et al.* (2006) *Spine* **31**:742-754. [4]T Chujo *et al.* (2006) *Spine* **31**:2909-17. [5]I Cowgill *et al.* (2006) *Trans ORS* #1185.[6]T Chujo *et al.* (2007) *Trans ORS* #267. [7]R Pichika *et al.* (2007) *Trans ORS* #243. [8]K Masuda (2008) *Trans WFSR* #81. [9]M Kawakami *et al.* (2007) *Trans ORS* #1202. [10]M Kawakami *et al.* (2007) Trans WFSR #38.

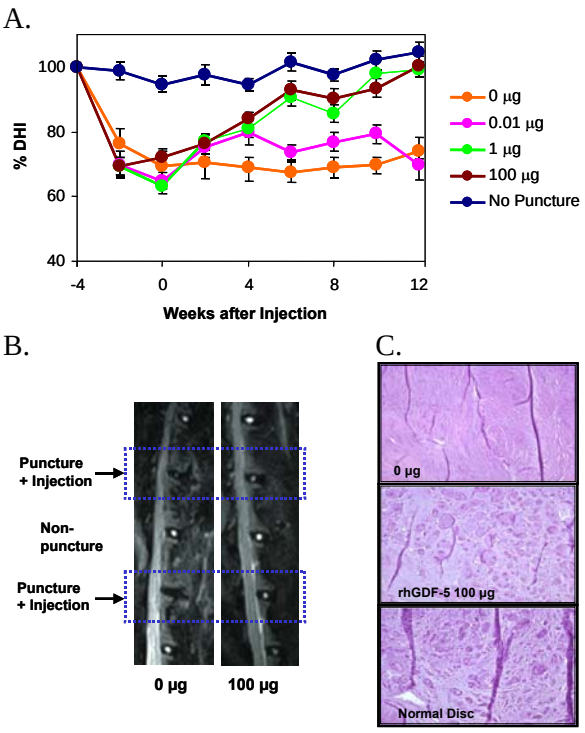

Fig 1. Effects of rhGDF-5 in the rabbit annulus puncture model[4]. (A) Disc height recovery following rhGDF-5 injection at t=0. (B) T2-weighted MRI images 12 wks after rhGDF-5 injection. (C) H&E histology of rabbit nucleus pulposus 12 wks after rhGDF-5 injection.

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Morphological changes of intervertebral disc cells in the porcine and human injured cervical spine following trauma.

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INTRODUCTION: The intervertebral discs are vital to the functioning of the spine in terms of its movement, load bearing and protection of the spinal cord. However, little is known about what influences the vitality and causes death of disc cells, or by what mechanism they may die. Trauma has been demonstrated to lead to apoptosis (so called programmed cell death) in articular cartilage and human thoracolumbar intervertebral discs. [1, 2] We have studied in detail the cell viability and manner of cell death in human cervical discs from patients who have undergone traumatic injuries to the spine with subsequent surgical stabilization.

METHODS: The anterior portion of intervertebral discs (annulus fibrosus (AF) and nucleus pulposus (NP)) and endplates from 44 patients with traumatic injuries to the cervical spine. They were examined histologically, using trypan blue exclusion and TUNEL staining, to assess cell viability and apoptosis, respectively. In addition, electron microscopy was used to study the ultrastructural morphology of the disc cells. Fractures were classified according to Magerl et al[3], depending whether they were mainly compressive, flexion or rotation injuries. Similar studies of disc and endplate were undertaken on porcine cervical spines, 0-24 hours post mortem.

RESULTS: Electron and light microscopy showed that up to 75% of human disc cells die within the first 24 hours of trauma, mainly by necrosis. Similar results were seen in pig discs post mortem. Two morphologies, previously not reported in the disc, were also seen, particularly in human discs with compressive fractures (A-, B1+2-fractures). These were: (i) chondroptosis, where cells have patchy, condensed chromatin and vacuoles but no true apoptotic bodies and (ii) 'ballooned cells', with poorly visualized, homogenous chromatin, in large cells often containing much glycogen (Fig.1a+b).

Fig.1a Chondroptotic cell (iAF) A3.3. Fracture

Fig.1b Balloon cell (iAF) A3.3. Fracture

Porcine samples revealed comparable rates of apoptosis and chondroptosis as fractures with less compression, but no ballooned cells.

DISCUSSION & CONCLUSION: Traumatic injuries of the human cervical spine lead to rapid changes in disc cell morphology and cell death, particularly via necrosis. The type of fracture and load appears to influence the type of cell death. This study describes for the first time an alternative form of cell death in discs, chondroptosis, in addition to a novel morphology for disc cells, 'balloon cells'. Balloon cells have been described previously in articular cartilage.[4] Interestingly tumour cells, such as in multiple myeloma, present a similar homogeneous nucleus and are known to be very active with a high rate of RNA synthesis, mitosis and enhanced protein synthesis. Whether balloon cells in the disc would be as active as such tumour cells, must be considered and investigated. Similarly, how altered disc cell morphology might influence the survival or degeneration of the disc and whether disc cells and disc matrix will partially recover following trauma remains to be investigated.

REFERENCES: [1]Tew SR, Kwan APL, Hahn A, Thomson BM, Archer CW. (2000) Arthritis& Rheumatism 43(1):215-25.
[2]Heyde C-E, Tschoeke S, Hellmuth M et al. (2006) BMC Clinical Pathology 6: 5-14.
[3] Magerl F, Aebi M, Gertzbein SD et al. (1994) Eur Spine J 3:184-201.
[4]Ghadially FN. (1997) Ultrastructural Pathology of Cell and Matrix. Butterworth-Heinemann.

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Effect of Limited Nutrition on Intervertebral Disc Cells Under "Physiological" Loading – A 21 Day Culture

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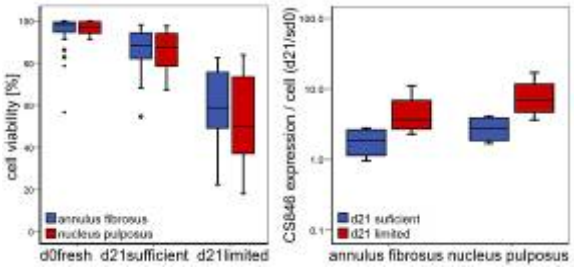
INTRODUCTION: The underlying causes of disc degeneration are multifactorial. Besides genetic factors and aging, limited nutrition is generally believed to be an etiological factor [1]. Although this effect has been demonstrated in cell culture, no investigations have been reported in actual discs. Recently; a novel in vitro system was developed for culturing whole intervertebral disc (IVD) explants with natural controlled loading through intact endplates (EPs). In this study, the mid term (21 days) response of disc cells, in their native extra cellular matrix (ECM), to nutritional challenge and "physiological" load was investigated.

METHODS: Discs were cultured for 21 days, under "physiological" loading i.e. diurnal axial load (0.2/0.6 MPa, 8/16 h) with cyclic load during the 0.6 MPa-phase (0.2 Hz ± 0.2 MPa, 2x4 h), and cultured in media with limited (lim=2 g/l) or sufficient (suf=4.5 g/l) glucose concentration. Cell viability was determined with LIVE/DEAD stain and cLSM and quantified using NIH/ImageJ software.

Relative gene expression of selected anabolic and catabolic genes was quantified by relative real-time RT-PCR ($^{\Delta\Delta}$Ct). Synthesis rate of aggrecan chondroitin sulfate was measured with CS846 epitope ELISA (IBEX) and normalized to number of living cell (from cell viability quantification).For statistical analysis all data were normalized to d0 and a Wilcoxon signed rank test blocked for sheep was used for cell viability, RT-PCR and GAG synthesis rate. For all statistical analyses, a $p < 0.05$ was considered significant.

RESULTS: Cell viability in fresh discs was ~90% and could be maintained for 21 days cultured under sufficient nutrition, cell viability dropped to ~50% when cultured under limited nutrition (Fig. 1A). Although CS846 content did increase with limited vs. sufficient glucose in the, this was not statistically significant (AF: suf=0.023 µg/cell (0.009-0.028) lim=0.041 µg/cell (0.019-0.105), NP: suf= 0.041 µg/cell (0.066-0.454), lim=0.128 µg/cell (0.1177-0.3087) values are medians, ± range; Fig.1B). In gene expression, no significant differences between culture conditions for AF or NP were detected (Data not shown).

Fig. 1: Boxplots of A) cell viability and B)



CS846 content in the annulus fibrosus (AF) and nucleus pulposus (NP) of discs cultured for 7 day.

DISCUSSION & CONCLUSIONS: Only cell viability and GAG synthesis rate followed the expected patterns of early degeneration. To induce severe signs of matrix breakdown 21 days of culture seems not long enough. To our knowledge, our study is the first to confirm Horner & Urban's (2001) cell work *in situ*. They found that cell viability stabilized to a new equilibrium after 2-4 days of culture. Here, the new equilibrium was reached within days and kept stable up to three weeks. In a next step we will investigate the synergistic effects of limited nutrition crossed with low (0.2Hz) and extreme high (10Hz) frequency loading in a seven day culture.

REFERENCES: [1]Urban,J.P.; Smith,S.; Fairbank,J.C. (2004) *Spine***29**:2700-2709, [2] Gantenbein,B.; Gruenhagen,T.; Lee,C.R.; Van Donkelaar,C.C.; Alini,M.; Ito,K. (2006), Spine31:2665-2673

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Pedicular screw fixation in osteoporotic vertebrae: intraoperative evaluation of local bone strength and bone augmentation via perforated pedicular screws.

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Posterior spinal fixation systems undergo internal constraints resulting in high load bearing requirement for the pedicular screw/bone interface. Different studies have proven that the screw performance is dependent on the bone quality. We have developed perforated screws for cement augmentation in osteoporotic vertebrae and analysed stiffness and screw migration.

To decide which vertebrae will need augmentation an intraoperative measurement of local bone density is required. Previous work of our group reported on laboratory evaluation of mechanical torque measurement as a method for the intraoperative quantification of bone strength and on the development of a related instrumentation for proximal femur fractures with DensiProbe™ Hip [1].

With the laboratory study presented here we intend to verify two different methods to evaluate the bone strength of trabecular bone in spinal instrumentation with pedicle screw techniques. In a specific protocol the global BMD of the vertebral body of lumbar vertebrae was compared with the mechanical strength of this anatomical region. The objective of this study was to evaluate two different measurement principles and to correlate the mechanical competence with BMD of the region around the pedicle screw tip.

Material

Mechanical measurement probes: Two principles of measurement techniques for intraoperative use to measure torque and indentation to breakaway of trabecular bone: Torque Measurement Tool (TMT) with a common data acquisition unit (HIOS HDM-100, Intechnik AG). Indentation Measurement Tool (IMT) with load cell (Burster Miniature Load Cell) and USB-Sensor Interface (type 9205) with the configuration software (MTS-Messtechnik GmbH). Hydraulic actuator MTS Mini Bionix 858. XtremeCT, Scanco Medical. 10 fresh frozen human lumbar vertebrae, level 4; BMD 56.1 to 117.0 mgHA/cm^3. 20 USS-II pedicle screws, D5.2mm, Synthes Inc. USS-II Instrumentation, Synthes Inc.

Methods

The vertebrae were mounted in respect to prone position, fixed in a vice. The handling was adapted to the standard Universal Spinal System (USS-II, Synthes Inc.) operation technique for segmental correction and stabilization. The instrumentation procedure was controlled with fluoroscopy. After preparing the screw canal a spine surgeon inserted the TMT and measured the breakaway torque. For the measurement with the IMT, a rod inside of the cannulted tip will be pushed into the trabecular bone to evaluate the resistance. After evaluation pedicle screws were inserted. The specimens were loaded in caudo-cranial direction according to studies of Reinhold [2] and Ferguson [3]. Therefore the pedicle screw heads were embedded in PMMA. Dynamic loading was applied to the cranial endplate through a metal ball on the end of the hydraulic actuator. A fluoroscope was used to determine the migration of the screws relative to the bone. Dynamic loading with a lower level of 20N was continuously increased from 100 to 800N at 0.035N/cycle until failure of the construct.

Results

High correlations were found between indentation load and breakaway torque ($r = 0.931$, $P < 0.001$). Also a high correlation could be shown between indentation load and BMD ($r = 0.929$, $P < 0.001$). Although the range of the torque measurement was less than 0.3Nm, a high correlation between breakaway torque and BMD could be established ($r = 0.924$, $P < 0.001$). Six out of ten specimens could be used for evaluation of the failure load. The evaluation of the indentation and failure load of the 6 specimens showed a significant correlation between these two parameters ($r = 0.968$, $P < 0.001$). Also a correlation between breakaway torque and failure load could be found ($r = 0.855$, $P = 0.030$).

A significance for the correlation between BMD and load to failure could also be determined ($r = 0.827$, $P = 0.042$).

Conclusions

For the evaluation of a predictive value for the cut-out resistance of pedicle screw, both tested tools seem to be appropriate. The holding strength of the pedicle screw was significantly influenced by both mechanical parameters, the indentation load and breakaway torque. The relation between BMD and load to failure could also be reflected. It is expected that the small number of specimens due to adaptations of the test protocol has an influence on the result. For a clear conclusion, which principle has to be considered as predictive value for the cut-out risk of pedicle screws, the final setup should be optimized.

References

1. Suhm et al. Arch Orthop Trauma Surg 2007
2. Reinhold et al. Spine 2006
3. Ferguson et al. Eur Spine J 2002

Survival of Bone Marrow Stromal Cells within Hydrogels: A comparison to Nucleus Pulposus Cells and Articular Chondrocytes

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INTRODUCTION: Bone marrow stromal cells (BMSC) are good candidates for cell based tissue regeneration strategies (e-g. for the Intervertebral disc) due to their proliferation and differentiation capacity. Most often BMSC are cultured in a 3D hydrogel matrix (e.g. alginate and agarose). For substantial tissue regeneration, survival of these cells inside these scaffolds is essential. Up to date, little is known about the fate of BMSC within these gels. We hypothesize that ACs and NP cells will thrive within these gels, in contrast to undifferentiated BMSCs, but that chondrogenic differentiation of the latter is expected to increase their survival.

METHODS: Bone marrow was harvested from five calves. BMSCs were isolated based on their capability to attach to the culture flasks. NP cells and ACs from the same animals were isolated by standard sequential pronase and collagenase digestion. Primary NP cells and ACs and passaged BMSCs (P2) were cast in 1.2% alginate or 2% ultra low gelling agarose (4 dia. x 2 mm discs) and cultured for 7 days in DMEM supplemented with 10% FCS, 50µg/ml ascorbic acid-2-phosphate, 1% non-essential amino acids (NEAA) and 1% Penicillin/ Streptomycin. Half the BMSCs were cultured in chondrogenic medium (DMEM supplemented with 1% ITS+, 50µg/ml ascorbic acid-2-phosphate, 1% NEAA, 1% Penicillin/ Streptomycin, 10ng/m $TGF\beta_1$ and 10^{-7} M Dexamethasone). At days 0 and 7, number of living cells, DNA, and chondrogenic gene expression was assessed. Cryosections were stained for collagen type II.

RESULTS: In 7 days, the amount of living NP cells and ACs increased in both scaffold types compared to day 0. In contrast, number of living BMSC decreased in both hydrogels, which was ameliorated to some extent by the addition of $TGF\beta_1$. DNA confirmed this trend (figure 1). NP cells and ACs both expressed the 'chondrogenic' genes and were stained positively for collagen type II. $TGF\beta_1$ pushed the BMSCs towards chondrogenic lineage by up-regulating their mRNA expression of collagen II and aggrecan. Furthermore, in samples cultured in the presence of $TGF\beta_1$ collagen type II was demonstrated.

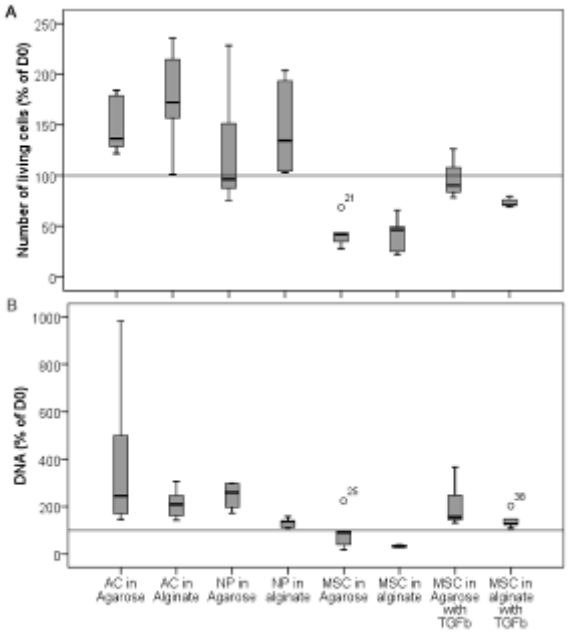

Figure 1: BMSC, NP cell and AC survival in agarose (Ag) and alginate (Al) at day 7, assessed by Live/dead (A) and DNA quantification (B)

DISCUSSION & CONCLUSIONS: Under the chosen conditions, these hydrogels appear to provide an appropriate environment for NP cells and ACs as indicated by their proliferation in culture and maintenance of phenotype. However, only a small fraction of the initially seeded undifferentiated BMSC could thrive in the same hydrogels under the same conditions. Since cell number and hence cell survival is a key factor for tissue regeneration, cell survival of BMSC within hydrogels should be improved. Addition of $TGF\beta_1$ to the cell culture resulted in chondrogenic induction and improved culture survival, but only to a limited extend. Hence, pre-conditioning of BMSCs may be a worthwhile strategy.

Clinical infection with fracture fixation – The Frutigen experience

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Introduction:
Infections are serious complications after internal fixation of fractures. Their incidence differs in relation to fracture location, is different in open and closed fractures and might be different in relation to the institution dealing with fractures and its socio-oeconomic environnement. The infection rate in the last 12 years in a small, mountain district hospital treating mostly monofractures resulting from winter- and summersport accidents is analysed and commented upon. Antibiotic coverage was administrated only in a selected group of patients.

Material and method:
Since 1995 all patients undergoing surgical fracture treatment were enlisted prospectively and called in routinely for a clinical and radiological control after 1 year. This so called AO-documentation and -control after 1 year has been pursued after 31st December 1999, date of discontinuation of routine AO documentation by the AO clinics.
Follow-up on the patients from abroad were either realised by a personal check-up by the respective surgeon or by a written report (including Xrays) from the medical doctor. Around 80% of the patients have been followed accordingly (1).
Antibiotic regimen: Patients with proximal femoral fractures, open fractures, delayed fixation and patients, that stayed in hospital longer than 6 hours before internal fixation and patients at risks (e.g. diabetes) were covered routinely with a single shot of a second generation cephalosporin. All other fractures were operated on without antibiotic coverage.

Results:
From January 1995 to December 2006 (12 years) 2467 osteosynthesis out of 13'922 interventions were carried out by 2 consultant surgeons. 241 external fixators (mainly distal radius fractures) have been excluded from the study. 21/ 2226 infections (0.94%) were recorded: 9 staph aureus, 4 staph epidermidis,, 2 MRSA, 1 bacillus cereus ,staph saprophyticus, enterococcus and clostridium perfringens each. 2 remained unknown. Infection rate in respect to fracture localisation: 7/299 (2.34%) proximal femur (3 DHS/ PFN, 4 endoprosthesis), 1/45 (2.2%) pilon tibial, 2/ 96 (2.1%) tibia plateau, 2/20 patella (10%), 4/ 223 (1.8%) tibia shaft, 1/71 (1.4%) femur shaft, 3/500 (0.6%) malleolar fractures, 1/254 (0.4%) hand fractures. Infections after internal fixation in other locations have not been recorded.

Discussion and conclusions:
The rate of infection after internal fixation of fractures in a rural hospital dealing mostly with simple monotrauma-patients was low and is comparable with literature (2,3). Patients at risk e.g. polytrauma patients resulting from traffic accidents were not treated at the institution due to the lack of an intensive care unit. Thus these results are not to be compared with results from big medical institution. Infection after proximal femur fractures were the most frequent, followed by proximal tibial fractures and tibial shaft fractures. The number of fixations of tibial pilon and patella fractures is (too) low for proper analysis. Infection rate after ankle and distal radius fractures were very low, respectively not recorded at all. Infections were mostly due to staph aureus, fortunately MRSA was not (yet) of importance. The antibiotic regimen applied may need reconsideration concerning tibial fractures.

References:
1) D. Heim, M. Schwab. Qualitätssicherung: Die AO Dokumentation am peripheren Spital. Swiss Surg 1999; 5: 276-280
2) CDC NNIS System, data summary Jan 92-June 2003, Am J Infect control 2003; 31: 481-98
3) Nast-Kolb D, Flohe S, Ruchholtz S. Akute Infektionen nach Osteosynthesel. Op Journal 2004; 20: 86-91

Improved detection and treatment of prosthetic joint infection

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INTRODUCTION: Although it is widely considered that aseptic loosening is the most common cause of failure of total hip replacement (THR), studies at Queen's University Belfast indicate that routine microbiological practice underestimates the incidence of infection[1.] By sampling directly from prostheses retrieved at the time of revision operation and adhering to strict anaerobic culture techniques the anaerobic bacterium *Propionibacterium acnes* is isolated as frequently as coagulase negative staphylococci. In addition non-culture detection using specific antibodies enables the detection of bacteria in culture negative samples[2]. We now present data relating to the study of prostheses retrieved from a further 125 patients and in addition examine bone samples.

METHODS: Samples were obtained from 125 patients undergoing revision operations for total hip arthroplasty at Musgrave Park Hospital, Belfast. Upon removal from the patient, samples were transferred into an anaerobic jar for transport to the laboratory where they were transferred to an Anaerobic Work Station (Don Whitley) for processing. Samples were placed in pre-reduced quarter strength Ringer's solution containing L-cysteine and then subjected to mild ultrasound treatment to dislodge adherent biofilm. Samples of the Ringer's solution (sonicate) were then spread plated onto blood agars and incubated aerobically and anaerobically. Sonicate samples were also incubated with antibodies that reacted with either *Staphylococcus* spp or *Propionibacterium acnes* and FITC-conjugated secondary antibodies and then viewed using a fluorescence microscope.

RESULTS: Of the 125 patients, 55 were male, 62 female and there was no gender information for 8 patients. The mean age was 70 years (34 to 91); age details were absent in 12 cases. The mean time-span of the implant *in situ* before failure and the need for revision surgery was 12.1 years (range of 1 year to 22 years). The study confirmed our previous data that handling of samples using strict anaerobic practice resulted in the isolation of *Propionibacterium acnes* as frequently as coagulase negative staphylococci. In addition, bacteria could also be cultured from bone samples after mild ultrasound treatment and detected by immuno-fluorescence microscopy (IFM). IFM revealed characteristic aggregates of bacteria that had been dislodged from adherent biofilm. Ninety-seven percent of the patients in the study had evidence of osteolysis.

DISCUSSION & CONCLUSIONS: Clearly, bacterial infection in THR is not restricted to bacteria growing in biofilm on the prosthesis surface, but may include biofilm colonisation of the adjacent bone. This has important implications for the potential role of bacteria in the osteolysis observed in these patients. These data also highlight the potential importance of the bacterium *Propionibacterium acnes* in the failure of total hip replacements. The potential virulence of this bacterium is becoming increasingly clear, with evidence of variable expression of putative determinants of virulence amongst different types[3].

REFERENCES: [1]Tunney MM, Patrick S, Gorman SP, Nixon JR, Anderson N, Davis RI, Hanna D and Ramage G (1998) Improved detection of infection in hip replacements: a currently underestimated problem *J Bone Joint Surg Br* **80**:568-72 [2]Tunney MM, Patrick S, Curran MD, et al Ramage G, Hanna D, Nixon JR, Gorman SP, Davis RI and Anderson N (1999) Detection of prosthetic hip Infection at revision arthroplasty by immunofluorescence microscopy and PCR amplification of the bacterial 16S rRNA Gene. J Clin Microbiol **37:**3281-90. [3]Valanne S, McDowell, A, Ramage G, Tunney MM, Einarsson GG , O'Hagan S , Wisdom GB , Fairley D , Bhatia A , Maisonneuve J-F , Lodes M , Persing DH and Patrick S (2005) CAMP factor homologues in *Propionibacterium acnes:* a new protein family differentially expressed by types I and II. *Microbiol* 151, 1369-1379.

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Can we Influence the Risk of Infection by Implant Design Changes?

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Introduction: The development of an infection after implantation of a fracture fixation device is known to be influenced by design aspects of the implant such as the surface area available for colonisation, whether or not it creates dead space, bone contact area, compression, periosteal necrosis and the stability provided by the implant[1,2]. *In vivo* studies using implants of identical dimensions, only differing in material have shown that the implant material is also a factor that can affect infection rate for intramedullary nails and dynamic compression plates[3,4]. Of the commonly used orthopaedic implant materials stainless steel and titanium; stainless steel is associated with an increased infection rate in comparison with titanium for intramedullary nails and dynamic compression plates [3, 4]. Aside from bulk material differences, these implants also differ in surface topography, from the smooth electropolished surface of stainless steel implants to the non-polished microrough surface of titanium implants.
Polishing the surface of titanium and titanium alloy internal fixation plates can minimise unwanted soft-tissue adhesion in areas where tissues must glide and ease removal of screws and intramedullary nails in comparison with standard equivalents [5,6], with significant clinical benefit in certain situations. The effect surface polishing has on *in vitro* bacterial adhesion and *in vivo* infection rate has been uncertain to date. Therefore we have evaluated locking compression plates (LCP's) which are designed to minimise damage to the periosteum, with standard microrough surfaces and polished ones to ascertain the effect of polishing titanium upon sensitivity to infection.

Methods: The materials under investigation included standard commercially pure titanium (cpTi) and titanium aluminium niobium alloy (TAN) both in their microrough form, as used clinically. Electropolished stainless steel (EPSS), cpTi and TAN were also investigated as polished equivalents. The surfaces of these metals were characterised with respect to topography, chemistry and hydrophobicity. Bacterial adhesion to the same five materials was performed i*n vitro* and subsequently an i*n vivo* investigation of the infection rate of the same five material types were compared using standard AO LCP's in New Zealand White rabbits.

Results: The surface characterisation showed that polishing cpTi and TAN reduced surface roughness without a significant change in chemistry or hydrophobicity. The *in vitro* analysis of bacterial adhesion found that polishing TAN surfaces significantly decreased the amount of *S. aureus* adhesion compared to the microrough standard TAN. The standard TAN had a higher affinity to the bacteria compared to cpTi (polished or rough) or EPSS. With the *in vivo* study using LCP's composed of these same materials, we found that polishing the surface of fracture fixation implants does not influence *in vivo* infection rate for cpTi or TAN in comparison with EPSS.

Discussion and conclusions: The *in vitro* results indicated that standard TAN is more susceptible *in vitro* to bacterial adhesion than polished TAN; however, we did not observe an increase for the *in vivo* infection rate for standard TAN in comparison with polished TAN. The *in vivo* results show that polishing either cpTi or TAN does not increase infection rate with the stable internal fixation system (LCP). The material related difference previously shown for intramedullary nails and dynamic compression plates for EPSS and cpTi [3, 4] is not retained in the locked system. The locked system is designed to minimise damage to the periosteal vasculature and surface of the bone. Therefore polishing which has previously been shown to ease implant removal, by prevention of bony integration and to prevent gliding tissue damage, could be clinically implemented with LCP's and screws, since we show that it does not reduce infection resistance.

References:

1. U. Schlegel, S. Perren, *Injury* 37 S67 (2006).
2. P. Worlock *et al*, *Injury* 25 31 (1994).
3. C. Hauke *et al*, *Orthop Trans* 21 835 (1997).
4. S. Arens *et al*, *JBJSBr* 78 647 (1996).
5. RG. Richards *et al*., *EORS Conference*, O-136 (2000).
6. RG. Richards, *et al., Trans. 53rd ORS*, 1629 (2007)

Anti-microbial device-based approaches to implant-centered infection

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INTRODUCTION: Device-related infection remains an unresolved clinical problem with increasing numbers of patients across different device classes in different implant scenarios. Many types of materials designs and anti-microbial approaches continue to be levied against this problem, both in infection prophylaxis and in infection therapy. The increased use of implantable materials and increasing incidence of antibiotic resistant infection makes this problem compelling. This presentation reviews several recent approaches to impart medical implant devices with anti-microbial properties.

METHODS: Many different approaches have been historically used to counter device-related infection, including antibiotic lavages, locally tethered or released anti-microbials, device coatings, local electric fields and current applications, and newer approaches targeting bacterial adhesion mechanisms, communication pathways and virulence factors. These include:

- Immobilized antimicrobials
- Controlled release anti-microbials
- Non-adhesive device coatings
- Surgical antiseptic lavages
- Silver metallic and salt formulations
- Electroactive treatments
- Quorum sensing disruptors
- Nanotechnologies

Combination devices provide some new opportunities for innovation by allowing local antibiotic formulations to be released from established classes of implants.[1,2] Few strategies to date have shown much efficacy in vivo in humans despite promising in vitro anti-microbial efficacy and even some translation to animal implant models. New antimicrobial antibodies allow local and systemic delivery of anti-pathogen antibodies to neutralize infection at implant sites and exploit natural endogenous mechanisms of microbial clearance using immune system cascades.[3] Additionally, new tissue engineering strategies to out-compete bacterial colonization by pre-seeding biomaterials with cells do not appear to be effective.[4]

RESULTS: Selected results from orthopaedic prosthesis infection models in spine are reproduced below. The lapine spine implant model[5] is shown in Figure 1, with anti-microbial efficacy of locally delivered polyclonal human antibodies in these wound sites shown in Figure 2.[6]

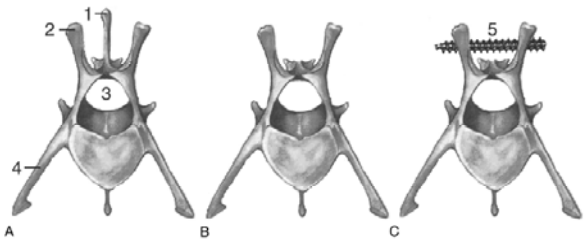

Fig. 1: Lumbar spine K-wire hardware infection model in rabbits for antimicrobial testing.[5]

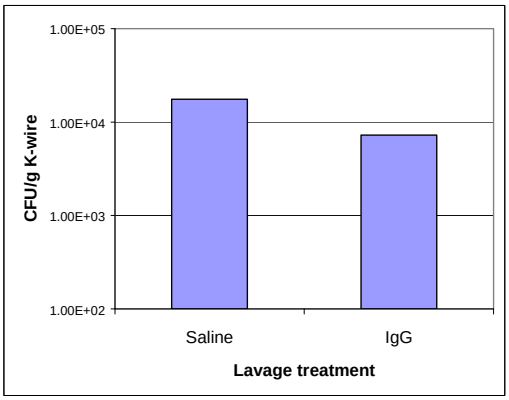

Fig. 2: Knock-down of MRSA in a spine K-wire hardware infection model in rabbits using human polycloncal antibodies.[6]

DISCUSSION & CONCLUSIONS: Antimicrobial strategies address important clinical challenges for increasing numbers of implanted devices and incidences of surgical infections. Unfortunately, most innovations fail to produce substantial improvements in clinical efficacy to date. Scientific issues involve inadequate evaluation methods, including problematic, non-predictive in vitro assays and also irrelevant animal models of infection with devices.

REFERENCES: [1]P. Wu, D.W. Grainger, *Biomaterials* 27, 2450 (2006);[2] I.A. Rojas, et al. *J. Control. Release* 63, 175 (2000); [3]D.W. Grainger, *Expert Opin. Biol. Therap.* 7, 1029 (2004); [4]R. Kuijer, et al. *Biomaterials 28* 5148 (2007). [5]K. Poelstra, et al., *Spine* 25, 406 (2000); [6]K. Poelstra, et al., *Tissue Eng.* 6, 401 (2000).

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Continuous real-time evaluation of microorganism growth kinetics & interactions with antimicrobial materials by isothermal micro-nano calorimetry (IMNC)

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INTRODUCTION: Cultured cell metabolism and growth processes produce heat measurable by IMNC.[1] The heat production rate (W=J/s) at any time t is the aggregate cell metabolic rate, and the amount of heat produced (J) between t_1 and t_2 is proportional to the number of cells produced. IMNC detects changes in heat production rate as low as 22 nJ/s--equivalent to a change of only ~10^4 in the number of active bacteria present. It is thus a non-invasive method for detecting and quantitating growth kinetics of bacteria in culture. IMNC can determine the effect of culture environment (incuding antimicrobials) on bacterial growth. After IMNC studies, the undisturbed culture specimens (cells, medium, solids) can be evaluated by any conventional means desired.

METHODS: The IMNC instrument is first equilibrated at a chosen temperature (e.g. 37ºC). Bacterial cultures of known types and concentrations (cfu/ml) are prepared conventionally. Studies are done in sterile 4 ml glass ampoules. Typically, 2.97 ml of a growth medium (with or without an antimicrobial) are added, followed by 0.03 ml PBS containing 10^4 cfu of bacteria. The ampoule is then septum sealed. Measurements start ~60 minutes later--after the ampoule is lowered first into the equilibration position in one of the instrument's calorimeters and then to the measurement position. The instrument used has 48 independent calorimeters, and can thus rapidly evaluate multiple culture variables and replicate specimens (TAM III-48, Waters/TA Inc., New Castle DE, USA). Heat produced by an ampoule can be monitored as long as desired, typically hours to days.

RESULTS: Our IMNC method rapidly determined whether a sample contained methicillin-resistant or susceptible *Staphylococcus aureus* (MRSA or MSSA).[1] *The determination could be made in ~ 4 hours vs. 24 hours by standard means.* In addition IMNC provided the MIC (minimum inhibitory concentration) of the antibiotic used.

We expanded this approach to determine MICs of 10 different antibiotics for 5 different surgically important bacteria.[2] Results correlated exactly with parallel standard assessments and reference values from the Clinical Laboratory Standards Institute (USA). *IMNC was simple and accurate.* At subinhibitory concentrations, growth curves (time histories of heat flow rate and aggregate heat) were reproducible for a given bacteria and medium. *Differences in the array of growth patterns for a group of media may be a means of bacterial identification.*

IMNC also determined the antimicrobial action of Ag^+ ions.[3] The MIC (no growth at 24 hours) of $AgNO_3$ was 8 mg/l. For an antimicrobial biomaterial (silica agglomerates containing silver particles d ~ 5-20 nm) the MICs were much higher (e.g. 250 mg/l for silica/20% silver) because of the reduced amount of Ag^+ available. *More importantly, IMNC showed something new. Subinhibitory concentrations of Ag^+ delay growth, but once it starts, initial concentration has no effect on the rate (Fig 1).*

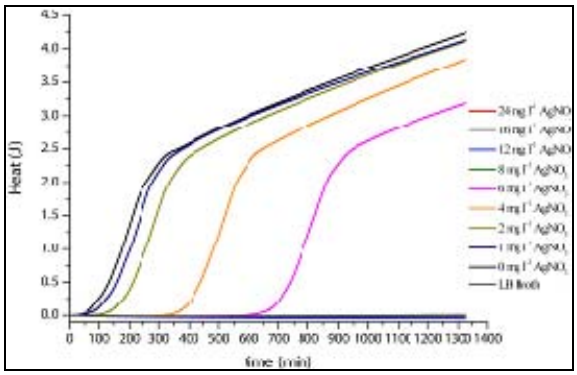

Fig. 1: E.coli growth in LB broth at 37°C. Aggregate heat as f(t) & $AgNO_3$ concentration.

DISCUSSION & CONCLUSIONS: Results suggest IMNC can be an important new tool for clinical and research microbiology. The power lies in its ability for continuous, real-time, quantitative monitoring of growth kinetics.

REFERENCES: [1] von Ah, Wirz, Daniels (2008) *J Clin Microbiol* (on-line 14/4/08). [2] von Ah, Wirz, Daniels (2008) *Report 301-MS4*, Velux Foundation, Zürich CH. [3] von Ah, Wirz, Pieles, Daniels (2008) *Abstract O9*, Ann. Mtg. Swiss Soc. for Biomaterials.

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ROLE OF THE CYTOSKELETON IN CELL LOCOMOTION.

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INTRODUCTION: Understanding the consequences of cell locomotion is central to understanding embryonic development and most of the body's healing processes as well as many disease conditions such as cancer. However, the role of cell locomotion in these processes is very complex and we don't yet even fully understand how cells move - though there has been rapid progress in understanding the molecular interactions that underlie cell movement. One problem that confronts us is the large variability between individual cells and the large variation in motile characteristics with time. Another is that the main supramolecular structures involved in cell movement and are not stable but are constantly disassembling, relocating and re-assembling: sometimes with a lifetime of only a few seconds. Our strategy is to develop new methods of microscopy and imaging to enable data from large numbers of cells to be gathered automatically and analysed by computer and to reveal the molecular activity within living cells as they move around.

METHODS: DRIMAPS (Digitally Recorded Interference Microscopy with Automatic Phase Shifting) is a technique we developed at the Randall Institute that that enables growth and motility data from large numbers of cells to be gathered automatically and analysed by computer. The images that it produces are calibrated maps of the distribution of cellular material (dry mass) within all cells in the field of view.

We have recently developed a method called FLAP (Fluorescence Localisation After Photobleaching) which uses laser light to label any specific protein molecule at any chosen location in the cell and then allows us to follow the fate of these labelled molecules as they relocate and participate in new structural configurations. Microinjection or transfection were used to introduce cDNA fusion constructs of β-actin with cyan (ECFP) and yellow (EYFP) fluorescent protein into cells. A laser-scanning confocal microscope allowed rapid localised bleaching and subsequent near simultaneous acquisition of cyan and yellow FP signals. Multi-channel detection allows the simultaneous acquisition of transmission phase contrast images to show cell morphology and interference reflection images to visualise areas of close contact between the cell and the substrate.

RESULTS: The FLAP method is beginning to tell us how cells protrude or push out a process which is the first phase in enabling them to crawl to a new location. The chief cytoskeletal protein involved in this process has long been known to be actin but the mechanism by which unpolymerised actin is transported to the leading edge has been poorly understood. FLAP was used to study actin dynamics within fibroblast-like cells and various malignant cell types. It has allowed us to follow both the fast relocation of monomeric G-actin and the much slower dynamics of filamentous F-actin simultaneously in living cells. We show several different behaviours of actin within the cell specific to different cell morphologies. By visualizing the ratio of bleached-to-total molecules, we found that actin was delivered to protruding zones of the leading edge at speeds exceeding five micrometers per second. Monte-Carlo modeling confirmed that this flow cannot be explained by diffusion and must involve active transport. Using fluorescence bleaching experiments, we have also discovered that actin polymerisation is confined to a very narrow band within 1 μm of the leading edge. We are now combining fluorescent methods with the DRIMAPS method to obtain estimates of the relative actin concentrations in different regions of the leading lamella.

REFERENCES:

Dunn, G.A., Dobbie, I. M., Monypenny, J, Holt, M R. and Zicha, D. (2002) Fluorescence Localization After Photobleaching (FLAP): a new method for studying protein dynamics in living cells. *J. Micros.*, **205**, 109-112.

Lai, FPL, Szczodrak, M, Block, J, Faix, J, Breitsprecher, D, Mannherz, HG, Stradal, TEB, Dunn, GA, Small, JV and Rottner,K (2008) Arp2/3 complex interactions and actin network turnover in lamellipodia. *EMBO J.*, **27,** 982–992

Zicha, D., Dobbie, I. M., Holt, M R., Monypenny J., Soong, D.Y.H. and Dunn, G.A. (2003) Rapid Actin Transport During Cell Protrusion. *Science* **300**, 142–145.

Confocal laser scanning microscopy in connective tissue research

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The confocal laser scanning microscope (CLSM) is a key tool in many areas of research where light microscope imaging, predominantly of fluorescent subjects, is important. Although there are low magnification applications, it is mostly used as a high magnification, high resolution light microscope and as such, in keeping with the interests of Dr Iolo ap Gwyn in whose honour this part of the of the meeting has been designed, could be regarded as being at the interface of light and electron microscopy. Fundamentally there are two forms of confocal microscope – one specialised for high speed "real time" imaging for viewing of rapid, dynamic events, such as calcium transients in cells, and another associated with high quality three dimensional imaging. Whilst the boundaries between these instruments have become blurred in recent years, it its the latter type and its applications that I shall deal with here.

The principle of confocal microscopy was developed in the 1950's, but its development was dependent on relatively recent advances in laser, detector and computer technology to take advantage of the technique. Fundamentally, CLSM is based on the fact that, especially at high magnifications, the plane of focus of the microscope is much smaller than the thickness of the specimen being examined. In conventional microscopy, this means that at any one focus position a small amount of specimen is in focus, but the overwhelming majority is out of focus. Since the image is made of both, the out of focus light severely degrades the image from the focal plane – a particular problem with fluorescence microscopy. The key to the confocal principle is that the optical arrangement of the illumination and image forming parts of the microscope. These result in only in-focus light being accepted by the microscope from the specimen – the so-called "optical sectioning" effect. The amount of light coming from the focal plane is small, and thus requires sensitive detection and amplification by photomultipliers, but the image is undegraded and very sharp. Also, the focal plane can be moved accurately through the depth of the specimen, taking an image at user-set intervals to give a stack of images of the specimen in perfect register. These images can be combined to give a sharp view of the whole specimen with no out of focus fluorescence. In addition, we can use the image stack to gain depth information: microscopy always gives the x-y plane; confocal imaging gives the z-plane as well, allowing 3-dimensional modelling of structures.

Applications of the CLSM in our laboratories have included cytoskeletal analysis of chondrocytes in tissue sections and explant cultures placed under mechanical loading, and 3 dimensional modelling of tendon cell shape, cytoskeletal and cell-cell junctional analysis in tissue sections and intact tendons - it is possible to use the optical sectioning properties of the instrument to gain structural information from underneath the surface of intact specimens in favourable circumstances. Recent and ongoing studies include cellular and cytoskeletal control of early matrix deposition in developing intervertebral disc, cornea and bone. All of these studies involve high magnifications, the production of optical section series and 3 dimensional modelling, sometimes of 3 fluorogens simultaneously. Current and future work will be directed towards understanding the finer details of what we can observe with our labelling, microscopical and computer modelling techniques in relation to ultrastructural studies of cell and matrix deposition and interaction.

The CLSM can be used as an analytical as well as structural tool, with appropriate use of fluorescent reagents and appropriate software packages. There are numerous reagents that allow concentration dependent imaging of ions, of intracellular pH and of particular organelles. In addition further dynamic aspects of cell and matrix and even protein interactions behaviour can be examined using techniques such as FRAP (fluorescence recovery after photobleaching: fluorescent signals can be bleached by high intensity laser irradiation; fluorescent molecules then diffuse in from the surroundings allowing fluorescence recovery) and FRET (flourescence resonance energy transfer: different fluorogens transfer energy when very close together, thus allowing stimulation of one with the wavelengths of light appropriate to the other; can be used for measuring distances between fluorescent tagged molecules). Indeed, applications are limited only by availability of fluorescent reagents and the imagination of the microscopist!

Cryo Electron Tomography -3D imaging at nm resolution

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INTRODUCTION: Electron tomography is a technique that uses a Transmission Electron Microscope to determine a three-dimensional (3D) structure from any given asymmetric object [1]. It is the leading method for studying 3D ultrastructure in the 5-20 nm resolution range allowing complex pleomorphic assemblies, such as organelles and cellular substructures, to be studied in their cellular context. This imaging method can be simply broken down into 3 steps. First, a series of two dimension projection images of the specimen are recorded and systematically tilted to different angles ($-70^{o}/+70^{o}$) in the microscope. Second, these individual images are aligned to a common origin and finally the projections are then backprojected to create a 3D representation of the sample [2].

For high-resolution imaging techniques, such as electron tomography, there is a need to maintain the position of the cell constituents at a scale finer than the desired resolution. Cryo-fixation is a method that exploits the high content of water in biological material and uses it as a fixative. Contrary to any fixation method that relies on diffusion, cryo-fixation is fast, does not change the state of the specimen and has the potential to preserve biological structures at the atomic level. Vitrification of the sample is the ultimate goal of cryo-fixation and two cryo-fixation methods are generally used in electron tomography [3]. Plunge freezing is suitable for small protein assemblies in suspension while high-pressure freezing (HPF) has been adapted to vitrify thick samples. Plunge freezing samples can be viewed directly but HPF samples must be sectioned, either in their frozen state (cryoultramicrotomy) or freeze substituted, resin embedded and sectioned at ambient temperature.

Successful imaging of the specimen invariably depends on choosing the appropriate specimen thickness and dose rate to provide a signal to noise ratio that is high enough to produce a meaningful image. For the tilt series required for electron tomography this dose must be partitioned between the many images taken at various tilt angles. Automation in modern microscopes, by computer control, minimises the electron dose on the sample during the tilt series as it automatically corrects for image shifts and focus changes during acquisition, without subjecting the sample to additional electron exposure.

Since goniometers are not perfectly eucentric, images of the tilt series must be translated and rotationally aligned for computation of the 3D reconstruction. Once the tilt images are aligned the 3D reconstruction can be computed using a back-projection algorithm. The resulting reconstruction can be viewed as sequential thin 2D slices through the sample or the most common approach is to use segmentation to extract features of interest from the volume. In some cases individual molecules (e.g structures resolved by x-ray crystallography) can be fitted to the features in the reconstructed volume [4]. This allows the rotational orientation of molecules to be defined within the structure, making tomograms a very elegant way of displaying ultrastructure in 3D.

REFERENCES: [1]D.De Rosier, A. Klug (1968) Nature **217**:130-134. [2] W. Baumeister (2002) *Curr Opin Struct Biol* **12**:679-684. [3] B. McEwen, M. Marko (2001) *J Histochem Cytochem* **49**: 553-564. G. Rh. Owen, D.L. Stokes (2007) An introduction to low dose electron tomography- from specimen preparation to data collection *in Modern Research and Educational Topics in Microscopy* (eds A Mendez-Vilas, J Diaz) Formatex, pp 939-950. [4] W. He, P. Cowin, D.L. Stokes (2003) *Science* **302**: 109-113.

In vivo and *in vitro* tomographic imaging of bone, implants and bioresorbable scaffolds

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INTRODUCTION: In the last two decades computed tomography has continuously increased its applications in basic research and in the healthcare field. With this technology, biological as well as non-biological structures can be visualized based on their ability to block the X-ray beam. From a series of two-dimensional cross-sectional images orthogonal to the scanning axis, 3D views of an object can be generated. This process enables subsequent volumetric representation of the structures and also the assessment of their morphological and densitometric properties.
The main goal of this article is to give a brief overview of experimental designs and evaluation methods used in our laboratories. The combination of standard protocols in computed tomography [1-3] with wide-ranging *in vivo* scanning capabilities and additional analytical methods (e.g. histology) underscores the remarkable potential of the technique.

***In vivo* and *In vitro* Imaging:** A peripheral clinical computer tomograph (XtremeCT) as well as a microCT system (uCT40, Scanco Medical, Brüttisellen, Switzerland) provide the raw data in a resolution range between 6 and 246µm. Several self-developed and custom-made adaptations have extended the spectrum of applications. *In vivo* scanning of sheep, rabbits and rats at different skeletal location have been carried out and longitudinal information was obtained. *In vitro* imaging was optimized and adjusted to allow scanning of bone samples with metallic implants and bioactive scaffolds.

Differentiated Evaluation: Repeatable and consistent data processing protocols are of key importance for results impact and relevance. New scripts and routines are continuously necessary to meet the demand of increasingly sophisticated experimental designs. Recent segmentation algorithms allow precise quantification of bone properties at different stages of healing as well as more accurate separation of biological and metallic resp. synthetic phase.

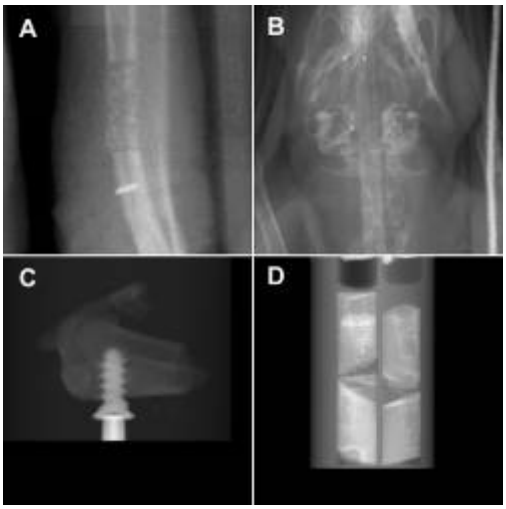

Fig. 1: Scout views of the regions of interest scanned in vivo (A, B) or in vitro(C,D). (A = critical radius defect in rabbit; B = calvarian defect in rabbit; C = implant osseointegration in ovariectomized rat; D = new bone formation with biomaterials in sheep)

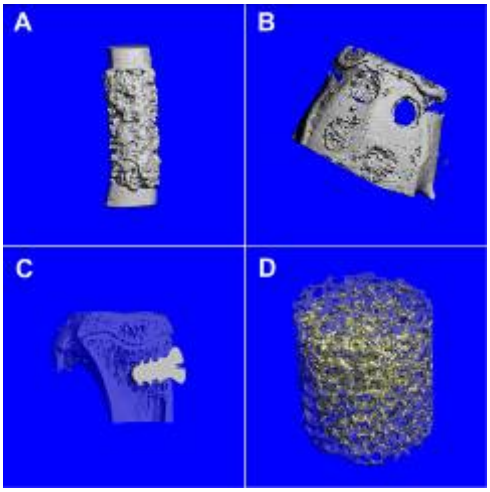

Fig. 2: 3D visualization of the samples shown in Figure 1. after appropriate data processing.

REFERENCES: [1] A. Laib et al (2000) *Med Biol Eng Comput* **38**:326-332. [2] J.A. Gasser et al (1995) *Bone* **17**:145-154. [3] G.H. van Lenthe et al (2007) *Biomaterials* **28**:2479-90.

ACKNOWLEDGEMENTS: The author would like to acknowledge the fruitful collaboration with AO-internal (Tissue Morphology Group, Experimental Surgery Group) as well as external groups (Institute for Biomechanics ETH Zurich).

Biological Scanning Electron Microscopy

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Scanning electron microscopy (SEM) has, to a great extent been regarded as the Cinderella of the electron microscopy world. The glamour and attraction of high resolution working at the limits of the technique have most often been associated with the transmission electron microscope (TEM). The SEM is most often regarded as being an instrument to produce low-resolution images for the role of illustrating some other, more high-powered, technique.

The development of the field emission gun scanning electron microscope (FESEM) enabled researchers to explore more fully the potential of this kind of microscopy to serve the needs of the research community. The increased resolution, offered by the use of a field-emission electron source has opened up a number of interesting possibilities to gain better information about biological samples.

Because the interaction between the beam electron and the specimen occurs at the atomic level then the emitted signal will reflect the consequences of such interactions. Traditionally, SEM imaging has been associated with the conversion of secondary emitted electrons (SE), from the specimen surface, into imaging information. To optimise such imaging it is necessary to coat the specimen with a thin coating of metal – by sputter coating. This process, in itself, will limit the spatial resolution available from the image.

Imaging using the backscattered electron (BSE) emission from the specimen – being the original beam electrons deflected out of the specimen by the atomic nuclei of the material in the upper layers of the material. The amount of deflection will depend upon the atomic number of the material. Thus, atomic-number contrasted images can be obtained. This has proven to be especially useful when performing immunocytochemistry studies.

By definition, the BSE signal level will be much lower than that of the SE. This means that more beam electrons are required. The FESEM provides such a facility and enables BSE imaging at a level that would not be possible with an ordinary SEM.

Another feature of BSE imaging is that, due to the higher energy of the electrons, the imaging signal can be emitted from considerable depths within the specimen – and not just from the surface layers, as is the case for SE imaging. We have exploited this feature in a number of ways, mainly involved with observing biological components stained or labelled with heavy metals. Such information can come from several microns within the specimen. Some interpretation is called for in order to understand the nature of what is observed in the images.

By ‘tuning’ the energy of the incident electron beam, by adjustment of the accelerating voltage, it is possible to control the depth from which the information is gathered. By reduction of the energy to a sufficiently low level it is also possible to ensure that unstained biological material will be sufficient to absorb the electron beam and hence create contrast. If the specimen is then placed upon a surface that is an efficient emitter of BSE and imaging is restricted to the BSE signal then, at the correct beam energy, that material can be imaged in the FESEM. Gold or Gold Palladium provides such a surface, but most other metals will also perform similarly.

Using this technique images of the cytoskeleton and bacterial biofilm have been produced, at high resolution, without resorting to either chemical fixation or staining of any sort.

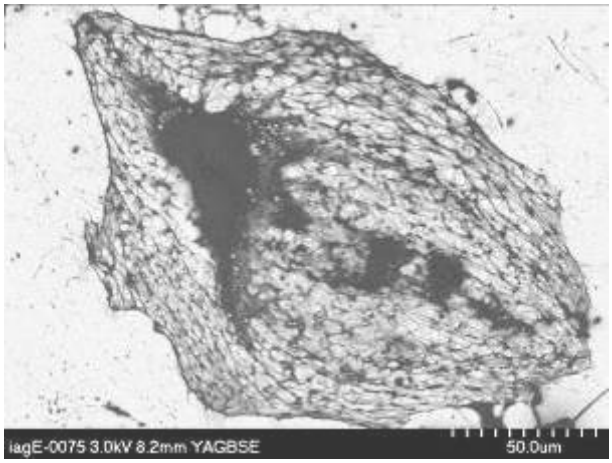

Fig 1. The cytoskeleton imaged without fixation or staining using BSE imaging in the FESEM.

ACKNOWLEDGEMENTS: To all the students and colleagues I have had the good fortune to collaborate with and to the AO foundation for financing many of the projects.

Inhibition of chondrocyte death at the wound edge enhances integrative cartilage repair.

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Introduction. Experimental wounding of articular cartilage leads to necrotic and apoptotic cell death at the lesion edge (1). It is hypothesised that the presence of healthy chondrocytes at the wound edge is critical for producing a repair tissue that is capable of integrating with the surrounding cartilage (2,3). Since repair of cartilage lesions often requires surgical debridement, associated chondrocyte death at the wound edges will be an inevitable problem. An earlier study has shown that short-term intra-articular administration of a caspase inhibitor decreases chondrocyte apoptosis at the wound edge and subsequently reduces cartilage degeneration following experimental osteochondral injury in rabbits (4). This study investigates whether inhibiting the cell death that occurs as a response of articular cartilage to wounding will result in enhanced cartilage integrative repair.

Materials and Methods. Full depth articular cartilage cores (6mm) were incubated for 3 hours in media containing inhibitors of necrosis (Necrostatin-1; 30µM; Biomol) or apoptosis (Z-VAD-FMK; 20µM; Calbiochem). At the end of this period, a 3mm inner core was cut and left *in situ* thereby creating a 'doughnut' model to study cartilage-cartilage integration. Cartilage was cultured for 2-weeks in media containing inhibitors with cultures being fed with fresh media and inhibitors every three days. Cartilage was analysed for necrotic (LDH release) and apoptotic (TUNEL) cell death, sGAG release (DMMB) and tissue integration (histology, immunohistochemistry, SEM).

Results. Within 48 hours of creating the wound, a significant ($p=0.003$) increase in LDH release was observed compared to unwounded cartilage. Treatment with Z-VAD-FMK significantly reduced the extent of LDH release ($p<0.02$) and apoptosis occurring at the wound edge. Necrostatin-1 reduced LDH release but the response was more variable ($p<0.058$). Treatment with either of the inhibitors reduced the level of sGAG lost into the media that resulted from wounding the cartilage. Toluidine blue staining of cartilage sections revealed significant integration of the wound edges in 'doughnuts' treated with Z-VAD-FMK. Necrostatin-1 improved integration but to a lesser extent. Immunohistochemistry revealed that in untreated doughnuts, there was an increase in pericellular staining for type III collagen extending out from the wound edge within the mid zone. Necrostatin-1 treatment resulted in an apparent increase in type I and III collagen whereas this was not observed in cartilage treated with Z-VAD-FMK. SEM analysis showed that Z-VAD-FMK treatment enhanced tissue integration.

Discussion. This study shows that treatment of articular cartilage with cell death inhibitors prior to wounding increases the number of viable cells at the wound edge, prevents matrix loss and results in a significant improvement in cartilage-cartilage integration.

References.

1. Tew SR, Kwan AP, Hann A, Thomson BM, Archer CW 2000 The reactions of articular cartilage to experimental wounding: role of apoptosis. Arthritis Rheum 43(1):215-25.
2. Huntley JS, McBirnie JM, Simpson AH, Hall AC 2005 Cutting-edge design to improve cell viability in osteochondral grafts. Osteoarthritis Cartilage 13(8):665-71.
3. Janssen LM, In der Maur CD, Bos PK, Hardillo JA, van Osch GJ 2006 Short-duration enzymatic treatment promotes integration of a cartilage graft in a defect. Ann Otol Rhinol Laryngol 115(6):461-8.
4. Dang AC, Warren AP, Kim HT 2006 Beneficial effects of intra-articular caspase inhibition therapy following osteochondral injury. Osteoarthritis Cartilage 14(6):526-32.

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CELL SOURCES FOR CARTILAGE TISSUE ENGINEERING

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Adult stem or progenitor cells have now been detected in most tissues of the body, including those relevant to the musculoskeletal system – bone marrow, bone, periosteum, cartilage, synovium, adipose and muscle. The cells of most tissues have been shown to have at least chondrogenic, osteogenic and adipogenic differentiation potential, and in some tissues, certain subfractions with greater differentiation potential are now described. Despite these advances, the relationship between the various cells of the different tissues is unclear, as is their relative importance in the field of regenerative medicine. There still remains a need for specific markers for these stem cell populations. Furthermore, to harness the therapeutic potential of stem cells for the treatment of skeletal pathologies, novel technologies need to be developed to isolate these cells effectively and efficiently.

Comprehensive comparative analyses, in vitro and in vivo, of the differentiation capability of the various adult stem cell populations are still lacking. Thus, it is still unclear which particular cell source provides the cells most applicable to a given tissue therapy. Comparisons of this type are also needed to address the notion that sorted populations will provide a better differentiation result in either ex vivo or in vivo tissue engineering and tissue regeneration. Indeed, it has to be considered that the sorted populations may actually be less useful because other cell types present in less purified populations may be beneficial for a given differentiation process to succeed.

Complicating the analysis done to-date is evidence that age- and gender-related differences may also play a role in the relative effectiveness of the isolated cells. For autologous human cell therapies, an added complication is the health status of the patient, which some evidence suggests can influence the number and potency of the stem cells isolated from various tissues. For this reason, several efforts are underway to isolate human stem cell populations that can be used as universal donors, taking advantage of the immune privilege that has been reported for these cells.

Even with optimized isolation and expansion methods for these cells, the field of stem cell-based therapies for skeletal tissues still faces many other challenges. We have yet to develop appropriate tissue engineering or tissue regeneration strategies that can be utilized in the clinical setting. The early promise shown in the preliminary studies of skeletal tissue engineering has been tempered by the realization of the complexity of stem cell interactions within implants, and with host tissues, which must be understood if we are to provide effective regenerative tissues for skeletal pathologies.

Scientists have developed a host of in vitro systems to investigate these issues. For example, our laboratory developed the in vitro system now widely used for the chondrogenic differentiation of stem cells. Variations on this system have been developed for different cell sources and applications. The system was originally developed in a scaffold-free format. However, for tissue engineering of implants on the scale needed for human cartilage pathologies, scaffold-based stem cell chondrogenesis has been widely investigated.

It was quickly appreciated application of biomechanical forces to the developing tissues in vitro would influence the composition and organization of the implants produced. Thus, the mechanobiology approach to skeletal tissue engineering has evolved. However, with the increasing sophistication comes increased cost. Despite the challenges, some of the first products of this field are now making their way into clinical trials.

Synovial Fluid Stem Cells: A Potential Cell Source for Cartilage Tissue Engineering

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INTRODUCTION: Adult mesenchymal stem/progenitor cells (MSCs) are potentially useful for engineering cartilage tissue because of their ease of proliferation and good chondrogenic capacity given the appropriate diffentiation signals. However, chondrogenic cells derived from differentiating MSCs isolated from bone marrow have been reported to undergo hypertrophy and mineralization in vivo[1]. Recently Jones et al[2] have demonstrated the presence of mesenchymal stem cells (MSCs) synovial fluid of patients with arthritis and normal SF fluid. The aim of this study was to isolate SF-derived MSCs from normal SF and human osteoarthritic SF and investigate their utility for cartilage tissue engineering using PGA scaffolds.

METHODS: MSC cultures were isolated from synovial fluids and cultured for at least 24 population doublings prior to conventional *in vitro* functional assays of chondrogenesis, adipogenesis and osteogenesis. Cultures of bovine chondrocytes were used as for a comparator. Scaffolds of PGA (Cellon, 5mm diameter, 2mm thick) were seeded with 2 x 10^6 MSCs or chondrocytes[3]. Some constructs were cultured in Dulbeccos Modified Eagle's medium, 3% FCS, 10 mM HEPES, non-essential amino acids, 1 mg/ml BSA, insulin/transferrrin/selenium,10^{-7}M dexamethasone, penicillin and streptomycin, 10 ng/ml transforming growth factor β_1 (TGF β_1) for the complete incubation period. Others were incubated with TGF β_3 and TGF β_1. Polyclonal MSC cultures were also pre-cultured in alginate gel for 14 d in medium supplemented with TGF β_1 prior to seeding on PGA. Constructs were mounted in OCT, and frozen sections taken for analysis of the extracellular matrix. Collagen I, and II were detected immunochemically. Proteoglycans were detected as glycosaminoglycans (GAGs) and localized using Alcian Blue or Toluidine Blue and quantified using dimethylmethylene blue.

RESULTS: SF-derived MSCs readily seeded onto the PGA scaffolds (96%). Immunohistochemical staining indicated all constructs produced some extracellular matrix with deposition of collagen II and proteoglycan. However, the SF-MSC constructs yielded less extracellular matrix (ECM) than chondrocyte constructs using cells of a similar passage number (571-801 µg sGAG/construct, n=4 vs 80-130 µg sGAG/construct, n=7).This matrix also had a relatively immature morphology compared to constructs of primary chondrocytes cultured under the same conditions. No mineralization was detected in any constructs. Initial experiments of pre-incubation of polyclonal MSCs in alginate gels before seeding on the PGS yielded larger constructs (28 mg, vs 12 mg)containing higher levels of GAGs (173µg sGAG/construct vs 111µg sGAG/construct). Treatment of constructs with TGF β_3 in addition to TGF β_1 also enhanced the amount of ECM produced by clonal SF-MSC constructs.

DISCUSSION & CONCLUSIONS: SF-MSCs formed immature chondrogenic constructs on PGA. The chondrogenic capacity of these stem cells was enhanced by treating the constructs with TGF β_3 in addition to TGF β_1 or by a short pre-incubation in alginate gel and exposure to TGF β_1 prior to seeding onto PGA. The results suggest that synovial fluid represents a potentially attractive source of MSCs which may have utility for cartilage repair therapies in trauma and arthritis.

REFERENCES: [1] Pelttari K et al. Arthritis and Rheum 2006;54:324-3266. [2]Jones EA et al. Arthritis And Rheumatism 2004;50:817-827. [3]Crawford A. and Dickinson S.C. Methods Mol. Biol. 238:147-157, 2004.

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Articular Chondroprogenitors as tools for cartilage tissue engineering

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INTRODUCTION: The use of stem/progenitor cells whose progeny have the capacity to form good quality cartilage matrix has been targeted as a therapeutic goal for the treatment of large defects of articular cartilage that result from serious trauma or widespread osteoarthritic lesions. Previously we reported the isolation and characterisation of a progenitor cell population that resides in the surface layer of neonatal bovine articular cartilage [1]. Here, we extend our observations by assessing progenitor cell chondrogenic potential as a function of their ability to elaborate a cartilage matrix in pellet cultures, maintenance of *sox9* expression, analysis of telomere length and telomerase activity following long-term clonal expansion.

METHODS Isolation and cultivation of chondroprogenitor clones. Chondroprogenitor cells were isolated on the basis of differential adhesion to fibronectin as previously described by Dowthwaite *et al* (2004) [1]. Isolated chondrocytes were subjected to differential adhesion on fibronectin-coated 60 mm dishes for 20 minutes in 4 mls 1:1 DMEM/F12 plus 10% foetal calf serum (Invitrogen, UK) at a concentration of 700 cells ml^{-1}. Colonies of >32 cells (chondroprogenitor clones) were isolated using cloning rings. Full-depth and superficial zone cells were enzymatically isolated using surgical dissection from the MCP joints Telomere lengths of samples were detected using the TeloTAGGG telomeric length assay kit (Roche Diagnostics, Sussex, UK). Comparative quantitative analysis of telomerase activity in samples was performed using a previously validated RTQ-TRAP methodology [2]. The ABI Prism TaqMan quantitative polymerase chain reaction (qPCR) system (Applied Biosystems, CA, USA) was used to study the relative expression levels of collagen type II, aggrecan, *sox9* and Notch-1 between different cell populations.

RESULTS: Cloned chondroprogenitors exhibited exponential growth for the first 20 population doublings (PD), then slower linear growth with evidence of replicative senescence at later passages. Mean telomere lengths of exponentially growing chondroprogenitors were significantly longer than dedifferentiated chondrocytes that had undergone a similar number of PD ($P<0.05$). Chondroprogenitors also had 2.6-fold greater telomerase activity and maintained *sox9* and Notch-1 mRNA expression whereas in dedifferentiated chondrocytes there was little or no detectable expression of these genes. Chondroprogenitors were induced to differentiate into cartilage in 3D pellet cultures, immunological investigation of *sox9*, Notch-1, aggrecan and PCNA expression showed evidence of co-ordinated growth and differentiation within the cartilage pellet.

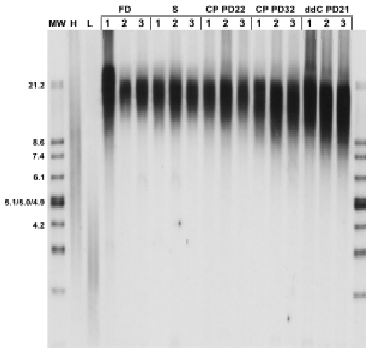

Fig. 1: ***Telomere length analysis of chondrocytes and chondroprogenitor clones. A.*** *Southern blot analysis of telomere lengths of restriction enzyme digested DNA of full depth articular cartilage chondrocytes (FD), surface zone chondrocytes (S), chondroprogenitor clones (CP) that had undergone ~22PD or ~32PD, and dedifferentiated chondrocytes (ddC, ~21PD).*

DISCUSSION & CONCLUSIONS: Maintenenace of telomerase activity, Notch-1 and *sox9* gene expression distinguish clonal chondroprogenitor cells from dedifferentiated chondrocytes. When placed in chondrogenic medium chondroprogenitors appear to engage in co-ordinated growth and differentiation in stark contrast to dedifferentiated chondrocytes that exhibit disregulated growth.

REFERENCES: [1]Dowthwaite *et al, J Cell Sci.* 2004 Feb 29;117(Pt 6):889-97 [2] Hou *et al. Clinical chemistry* 2001, 47:519-524.

DO WE REALLY NEED CARTILAGE TISSUE ENGINEERING?

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INTRODUCTION: Typical cell-based cartilage repair techniques rely on the implantation of chondrocytes in suspension (ACI) or seeded on specific scaffolds (MACI). In the medium term, the resulting repair tissue is often fibrocartilaginous, lacking the biochemical and mechanical properties of hyaline cartilage and thus possibly affecting the durability of the clinical outcome. Engineered cartilaginous tissues, where cells are embedded within a hyaline-like extracellular matrix, could not only have superior handling at the time of implantation, but also provide the appropriate cues to induce hyaline cartilage regeneration. In this lecture, we will review a few examples generated using in vitro and in vivo pre-clinical models, addressing the possible advantages of grafting pre-developed cartilaginous tissues.

ECTOPIC DEVELOPMENT

Monolayer-expanded human articular chondrocytes (HAC) were seeded into Hyaff-11® meshes (FAB). Constructs were directly implanted subcutaneously in nude mice for up to 8 weeks or pre-cultured in media promoting proliferation or differentiation for 2 weeks prior to implantation. As compared to direct implantation of freshly seeded scaffolds, pre-culture of constructs in *differentiating medium*, but not in *proliferating medium*, supported an enhanced in vivo development of engineered cartilage, as assessed histologically, biochemically and biomechanically[1]

RESPONSE TO INFLAMMATORY SIGNALS

Monolayer-expanded HAC were cultured in pellets for 3 or 15 days in chondrogenic medium and assessed for the production of anabolic and pro-inflammatory cytokines in response to IL-1β treatment. By increasing culture time, cells released lower amounts of IL-8 and MCP-1 and higher amounts of TGFb-1. As compared to HAC cultured for 3 days, those cultured for 15 days responded to IL-1β releasing lower MMP-1 and MMP-13 amounts[2]

RESPONSE TO MECHANICAL LOADING

HAC expanded in monolayers were seeded on different polymeric scaffolds, cultured for different durations and exposed or not to dynamic deformation. Upon application of compression, changes in glycosaminoglycan (GAG) synthesized, accumulated, and released were significantly positively correlated to the GAG content of the constructs prior to loading, and resulted in improved tissue quality only in the most developed tissues[3,4].

ORTHOTOPIC IMPLANTATION IN GOATS

Engineered cartilage was generated by culture of autologous articular chondrocytes in Hyaff-11® meshes for 2 days, 2 weeks or 6 weeks and implanted on top of hydroxyapatite/Hyaff-11® sponges into goat osteochondral defects for 8 months. Additional experimental groups included defects that left untreated or treated with cell-free scaffolds. Modified O'Driscoll scores indicated poor cartilage repair for untreated and cell-free treated groups. Instead, the use of cells improved cartilage repair, and grafts cultured for 2 weeks performed better than those generated in 2 days[5].

CONCLUSIONS: The reviewed studies indicate that, as compared to scaffolds freshly seeded with cells or immature tissues, more mature engineered cartilaginous tissues would have the potential to support superior cartilage repair. This could be due to a combination of (i) the intrinsic capacity to develop, (ii) the resistance to inflammatory processes, (iii) the modality of transduction of loading to cells. These pre-clinical evidences prompt for randomized, prospective trials where the performance of mature engineered cartilage grafts is compared to that of typical ACI or MACI techniques.

REFERENCES

[1]Moretti *et al.*, Tissue Engineering 2005;11:1421-8.

[2]Francioli *et al.*, Tissue Engineering and Regenerative Medicine International Society (TERMIS) Porto 2008.

[3]Demarteau *et al.*, Biochem Biophys Res Commun 2003;310:580-8.

[4]Candrian *et al.*, Arthritis Rheum 2008;57:197-208.

[5]Miot *et al.*, International Cartilage Repair Society (ICRS) Warsaw 2007.

SUCCESS/FAILURE IN ORTHOPAEDIC CELL ENGINEERING

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Science begins and ends with observation. Surgeons observe cell activity in normal healing, interfere with surgery, and increasingly move selected and culture expanded cells to injured areas. What observations can the clinician bring to the scientist? In orthopaedics, arthritis and fractures are our main problems. How can cell engineering help? Several groups have engineered new bone with MSCs, but skepticism remains over the ability to form cartilage with chondrocytes. Are the successes and failures due to biomechanical, biological or a 'choice of cells' issue? The main debate in the management of chondral defects is focused on the difference between adding cultured chondrocytes and techniques that enable and encourage natural healing. Clinical trials suffer from varied ingoes and fuzzy outcomes. The main debate in the management of chondral defects is focused on the difference between adding cultured chondrocytes and techniques that enable and encourage natural healing. Clinical trials suffer form varied ingoes and fuzzy outcomes.

The best test of the original ACI technique1 to date remains the study by Knutsen2. This Norwegian randomized trial was run by surgeons who had never before performed ACI, and each surgeon only completed a few cases in the trial. The report at two years was of only a significant difference in one of 8 parameters measured, supporting microfracture over ACI3. A trend to better histology was noted in the ACI group, but there were difficulties in quantifying the histology. At 5 years the results (to be published in March 2008) are of equivalence in failure rates and symptoms. About 20% of patients have had further operations. The failures have been in those with poor histology. There are only 80 patients in that trial, but excellent follow-up is a tribute to the team and the patients involved.

A Belgian company Tigenix have developed a method of selecting the best cells in a patient's culture for the formation of cartilage, they believe. They have run a prospective randomised trial. Histology was assessed blindly using a newly developed score, and reports an improved histology for ACI over microfracture3. I place this as the most important report of recent years in this field. Taken with the observation from Norway that the histology predicts failure, this is evidence for ACI.providing better outcomes.

ACTIVE is a prospective randomized trial, sponsored by Keele University, funded by MRC and the NHS of UK, with a ten year follow-up. This trial is pragmatic: designed to be 'real world' and adapt to technique developments. The entry criteria are broad, setting ACI techniques against 'best alternative', and this allows generalization of the results following the trial. 210 patients are now enrolled. This makes it the largest randomised clinical trial that is testing the original autologous chondrocyte implantation (ACI) technique, and the plan is to continue recruitment up to 480 patients. Chondrocytes loaded in collagen are included in the cell culture arm, and recently AMIC as a 'best alternative'. Norway and 12 centres in the UK do the hard work of recruiting, with Stanmore in London recently joining.

Cost-effectiveness is an important measure in a health service where costs are rising. A study from Aberdeen reported that if ACI is just 10 to 20% better than an alternative at 10 years, then the extra costs of cell culture are cost-effective4. This sets the target for clinical trials as 10 year follow-up.

Are the failures of ACI due to failure to regenerate the underlying bone? Is this a problem of inappropriate cells being present? In non-union co-workers at Oswestry, and also in Kobe Japan and in Germany5 have identified a 'non-union cell' present where bone fails to heal. Some patients with persisting pain have intense remodellng in the underlying bone. A technique of cell implantation over a large bone plug is giving good pain relief. This indicates that a biological joint replacement will need to replace both bone and cartilage.

Some preliminary work of the Myjoint EU programme will be presented where a new joint is planned to be formed in an 'endocultivation' bioreactor in the latissimus dorsi of the patient.

References:

[1]. Peterson, et al., Autologous chondrocyte transplantation. Biomechanics and long-term durability. American Journal of Sports Medicine 2002; 30:2-12; [2]. Knutsen, et al., A Randomized Trial Comparing Autologous Chondrocyte Implantation with Microfracture. Findings at Five Years. J Bone Joint Surg Am 2007; 89: 2105-2112; [3].Saris et al., Characterized Chondrocyte Implantation Results in Better Structural Repair When Treating Symptomatic Cartilage Defects of the Knee in a Randomized Controlled Trial Versus Microfracture Am J Sports Med 2008 36: 235-246; [4]. Clinical and cost-effectiveness of autologous chondrocyte implantation for cartilage defects in knee joints: systematic review and economic evaluation. Cummins,et al., Health Technol Assess 2005;9(47):1–98; [5]. Hofmann et al., Cell viability, osteoblast differentiation, and gene expression are altered in human osteoblasts from hypertrophic fracture non-unions.Bone. 2008 May;42(5):894-906.

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Integration Strength of Engineered Cartilage to Native Cartilage and Bone and Synthetic Substrate

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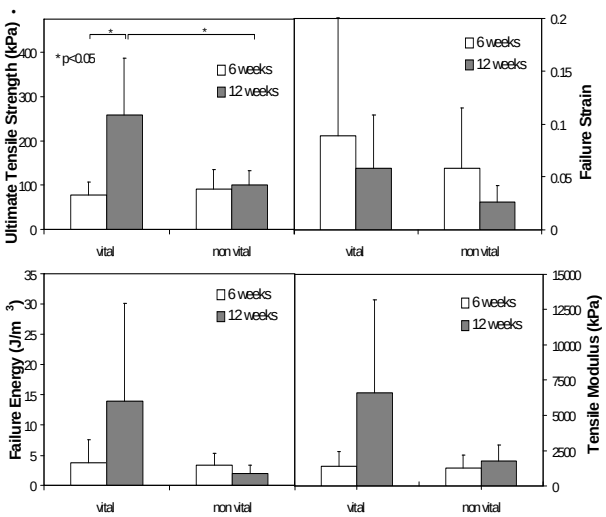
INTRODUCTION: The success of tissue engineering approaches for articular cartilage repair and regeneration relies not only on the ability of the chondrocytes to form new extracellular cartilage matrix, but also on the capacity of the new matrix to integrate with the existing cartilage surrounding the lesion and bone at the base of the lesion. The objective of this study was to investigate the integrative strength of engineered cartilage to: 1) native cartilage, 2) bone; and 3) and a synthetic porous polyethylene (PPE) substrate that could be used as part of a replacement osteochondral plug.

METHODS: Cartilage and bone discs (6mm) were made from swine tissues and PPE was commercially available. Swine chondrocytes were suspended in fibrinogen at a concentration of $80x10^6$ cells/cc and mixed with the same amount of thrombin forming a slowly polymerizing fibrin glue mixture. 150 μL of the fibrin glue-cell mixture was placed on one disc. Another disc was placed on top of the fibrin glue-cell mixture and allowed to gel. Control constructs were made using acellular fibrin glue or fibroblasts in the middle. The constructs were implanted into subcutaneous pouches in the backs of nude mice for 6 and 12 weeks. Randomly selected specimens were processed for histology, and the remaining specimens were stored at –80°C prior to biomechanical testing.

The mechanical integrity of the bonding of the new cartilage to the substrate was evaluated on an Enduratech spectrometer. The constructs were attached to plexiglass rods using quick-setting cyanoacrylate glue and mounted in the actuator of the Enduratech. Tensile displacements were applied at a rate of 10 μm/s to failure and the resultant loads were recorded. Sample displacements and loads were normalized to strain and stress by sample geometry and intergration (ultimate tensile) strength (σ_{UTS}), failure strain (ε_f), failure energy (E_f), and tensile modulus (M) were calculated from the resultant stress-strain curves.

RESULTS: Histology showed new cartilage matrix formed between the discs when chondrocytes were incorporated in the fibrin glue. No cartilage formed between discs when chondrocytes were omitted or using fibroblasts. For brevity, data using bone discs are shown (Figure). Integration strength (σ_{UTS}) was highest in 12 weeks samples as demonstrated vital bone. At 12 weeks, ultimate tensile strength was ~260 kPa, a 3-fold increase over that at 6 weeks ($p<0.05$) and 2.5 fold higher than in samples using devitalized bone ($p<0.05$). Similar trends were observed in failure energy and tensile modulus, with 12 weeks samples from vital bone larger than all other groups. There was no difference in failure strain between groups. The integration strength of neocartilage with native cartilage at 12 weeks was ~90 kPa and that with PPE was ~140 kPa.



DISCUSSION & CONCLUSIONS: The integration of an engineered cartilage implant to the existing bone and cartilage surrounding a lesion is critical for a stable and successful repair. These data demonstrate that engineered cartilage has the capacity to integrate with native cartilage and bone, as well as a synthetic nondegradable material. This model could simulate the integration of cartilage with the bone and cartilage surrounding a lesion and promote regeneration of the articular surface. Further studies will focus on understanding mechanisms that control process of integrative bone and cartilage repair employing tissue engineering strategies.

ACKNOWLEDGEMENTS: These studies were supported in part by the AO foundation.

The Effect of Sliding Velocity on Chondrocytes Activity in 3D Scaffolds

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INTRODUCTION: Sliding motion and shear have widely been recognized as important mediators for the synthesis of cartilage matrix and surface molecules. The specific contribution of the sliding velocity vector, however, has not been systematically addressed. This study investigated the effect of (i) the velocity magnitude and (ii) the motion shape on the response of bovine chondrocytes cultured in polyurethane scaffolds and subjected to oscillation of a ceramic ball over the scaffold surface or oscillation of the scaffold against the ball.

METHODS: A ceramic hip ball was pressed onto the cell-seeded cylindrical scaffold. Interface motion was generated either by reciprocating rotation of the ball about an axis perpendicular to the scaffold axis or by oscillation of the scaffold around its cylinder axis. The ball oscillated ±25° at 0.01, 0.1, or 1 Hz, resulting in surface velocity magnitudes of 0.28, 2.8, or 28 mm/s, respectively. To test the influence of the motion shape, these 'open' motion trajectories were tested against 'closed' trajectories in that the scaffold oscillated ±20° against the ball at 1 Hz, reaching the median velocity of 2.8 mm/s.

Constructs were loaded twice a day for one hour over 5 days. Unloaded constructs and constructs exposed to the static preload only served as controls. Gene expression of cartilage oligomeric matrix protein (COMP), proteoglycan 4 (PRG4, or lubricin) and hyaluronan synthase 1 (HAS1) and release of COMP, PRG4, and hyaluronan (HA) were analyzed.

RESULTS: Compared with statically loaded samples, COMP mRNA was increased already at 0.28 mm/s (Figure 1). At 2.8 mm/s, PRG4 and COMP release were also enhanced, while all measured parameters were significantly up-regulated at 28 mm/s. Using linear regression models, the magnitude of sliding velocity determined both gene expression and release of all target molecules.

Motion shape characteristics affected COMP, but not PRG4 and HAS1/HA. COMP mRNA expression was higher in constructs subjected to 'closed' motion trajectories, while the opposite was found regarding COMP release.

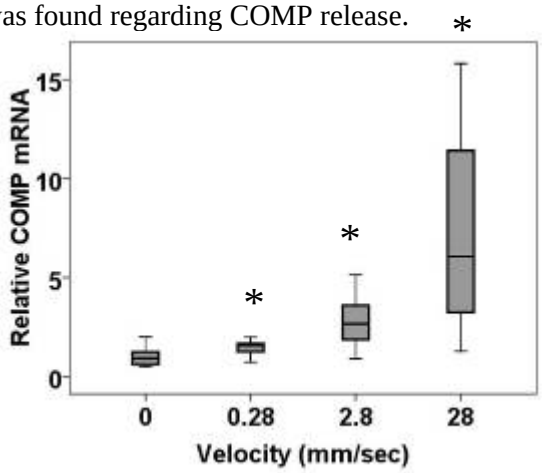

*Fig. 1. Relative mRNA expression of COMP in chondrocytes cultured in 3D scaffolds and subjected to different surface velocities. Expression normalized to unloaded controls. *p<0.05 vs. statically loaded constructs.*

DISCUSSION & CONCLUSIONS: Velocity magnitude is a critical determinant for cellular responses in tissue engineered cartilage constructs. Furthermore, the type of motion plays a role, too. However, these observations cannot be generalized, and there is a difference in the behavior of different molecules. The matrix protein COMP was most affected by both velocity magnitude and velocity profile. The apparent paradox regarding COMP gene expression and protein release can be explained as follows: The closed circular motion of the rotating scaffold induces pure shear without volume dilation to the surface. In addition, due to the closed trajectory shape, media is not exchanged with its environment. This disallows the transport of freshly synthesized molecules out of the contact area. Overall, for all investigated molecules, a certain velocity threshold appears to be necessary to induce a significant response. The specific motion type is of secondary importance. This should be considered in further studies investigating the effects of continuous or intermittent motion.

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Chondroitin sulphate motifs as biomarkers for the stem/progenitor cell niche in musculoskeletal tissues.

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INTRODUCTION: In the mid-1980's our laboratory produced and characterised several monoclonal antibodies [mAbs 3-B-3(-), 4-C-3, 6-C-3 & 7-D-4) that recognised unique 'native' sulphation motifs in chondroitin sulphate (CS) glycosaminoglycan (GAG) chains on connective tissue proteoglycans (PGs). These antibodies were shown to specifically locate CS-PGs in the peri-cellular regions surrounding putative sites where haemopoietic stem cells were undergoing lymphopoiesis in the Bursa of Fabricius of embroyonic chicks (Sorrell et al 1988, *J Immunol 140: 4263*; Caterson et al 1990, *J Cell Sci 97: 411*). In later studies, we also observed immunostaining for some of these mAbs [3-B-3(-) & 7-D-4] in chondrocyte clusters present in tissue sections from late-stage osteoarthritic cartilage from canine and human patients (Visco et al 1993, *Arthritis Rheum 36: 1718*; Slater et al 1995, *Arthritis Rheum 38: 655*). In a very recent study (Hayes et al 2008 *J. Histochem Cytochem 56: 125*) we have used these anti-CS GAG sulphation motif mAbs to identify proteoglycans with these specific GAG motifs in pericellular domains surrounding stem and/or chondroprogenitor cells located in the surface zone of hyaline articular cartilage.

METHODS: Our cartilage studies were performed using hyaline articular cartilage harvested from 1–2 week old bovine calves. Full thickness sections of this cartilage tissue were used for fluorescent and confocal immunohistochemical (IHC) analyses. In some instances these full depth cartilage pieces were carefully dissected into surface, middle and deep zone morphological regions and used for either 3D confocal microscopy analyses, extraction of proteoglycans followed by analyses using Western blot or Dot blot procedures or the isolation of chondrocytes from the different zones (using pronase & collagenase) for use with these mAbs in FACS analyses to sort and isolate chondro-progenitor cells for potential pluripotent cell enrichment in tissue engineering or tissue regeneration technologies. Sections from other musculoskeletal tisses (i.e. bovine tendon & developing rat intervertebral disc) and other animal organs (i.e. mouse gut & the developing chick eye) were also obtained and IHC analyses performed using these native CS GAG motif mAbs.

RESULTS & DISCUSSION: In our recent studies we have shown that our mAbs [3-B-3(-), 4-C-3 & 7-D-4] that recognise novel CS sulfation motif epitopes in GAG chains on proteoglycans can be used to identify metabolically distinct sub-populations of cells specifically within the superficial zone of hyaline articular cartilage, and that flow cytometric analysis can recognize these cell sub-populations. Fluorochrome co-localisation analysis suggest that these CS sulphation motifs are associated with a range of cell and extracellular matrix (ECM) PGs within the stem cell niche, that include perlecan and aggrecan, but not versican. We have also used several of these mAbs to identify stem/progenitor cells in different anatomical and functional regions of the tendon; i.e. where the tendon wraps around bone in compressed regions where the cells exhibit a more chondrogenic phenotype and also in the outer zones of the bovine tendon surrounding pericytes where vascularisation is present. In studies of the developing rat intervertebral some of these mAbs specifically recognise stem/progenitor cells at the interzone between the outer and inner anulus an also the boundary of the nucleus with the inner annulus, these results indicating their potential use for stem/progenitor cell identification and isolation in other musculoskeletal tissues. Interestingly, one of these mAbs (6-C-3) also immunostained the pericellular environment ("stem cell niche") in the crypts of the mouse gut and several mAbs the limbus of the chicken eye where stem cells reside.

CONCLUSIONS: The unique distributions of these CS motifs on PGs found within the pericellular micro-environment of superficial zone chondrocytes in cartilage is conistant with that reported from other studies (Dowthwaite et al 2004, *J Cell Sci 117: 889*) and they appear to identify early stages of stem/progenitor cell differentiation which is consistent with these molecules playing a functional role in regulating aspects of chondrogenesis. Collectively, this data suggests that these mAbs that recognise CS sulphation motifs can be used as biomarkers to identify stem cell niches in numerous tissues of the body and that they can be used for stem & progenitor cell isolation.

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Connexin43 expression in cartilage progenitor cells and its possible role in cell differentiation.

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INTRODUCTION: Recent research has identified the presence of a progenitor cell population in the surface zone of articular cartilage[1]. These cells have been shown to possess an extended cell cycle, be capable of forming large colonies from a single cell and can engraft functionally into a variety of connective tissues. It is thought these cells are required for the appositional growth of the tissue. During normal skeletal development, one of the earliest events seen is the formation of a dense cell mesenchymal cell condensation. It is thought that an increase in gap junction formation within this cell condensation is responsible for passing signals between cells and directing differentiation[2]. A novel non-invasive method for observing cell interactions *in* vitro has recently been developed using an ultrasound wave. When placed in the ultrasound wave trap cells have been shown to form 2D aggregates without altering the cells in any way[3]. In this study, the ability of the progenitor cells to express connexin43 and form functional gap junctions was examined.

METHODS: Initially cells were suspended in an ultrasound trap to create the formation of cell aggregates which were immunolabelled for connexin43. To determine if these connexin molecules were capable of forming functional gap junctions, cells were labelled with the gap junction permeable cell tracker CMFDA. Three groups of cells were used: surface zone cells (containing approximately 0.5% progenitors and 99.5% differentiated chondrocytes), a clonal progenitor population and surface/progenitor mix. In each case a 1:3 ratio of labelled:unlabelled cells was used.

RESULTS: Although the progenitor cells do express connexin on their surface, they are incapable of transferring dye between cells. Non-progenitor cells isolated from the surface zone of articular cartilage are able to form functional gap junctions almost immediately after aggregate formation. When these cells are mixed with unlabelled progenitor cells, dye transfer also occurs from the non-progenitor surface zone cells toward the progenitor cells

DISCUSSION & CONCLUSIONS: It has already been demonstrated that connexin expression and gap junction formation are vital for articular cartilage development. This study shows that populations of progenitor cells isolated from the surface of bovine articular cartilage express connexin43 in culture. After aggregate formation, connexin43 expression occurs predominantly at regions of cell-cell contact. Despite this the cells are incapable of forming functional gap junctions with other progenitor cell populations after one hour in the ultrasound trap. Mixing the progenitor cells with terminally differentiated cells does however result in dye transfer, suggesting that they are capable of communicating with more differentiated cells types. It is possible that signals transferred by mature cells within the surface of cartilage could be a signal for the progenitor cells to undergo differentiation.

REFERENCES: [1]G.P. Dowthwaite, J.C. Bishop, S.N. Redman, I.M. Khan, P. Rooney, D.J. Evans, L. Haughton, Z. Bayram, S. Boyer, B. Thomson, M.S. Wolfe and C.W. Archer (2004) *J Cell Sci* 117:889-97 [2]B. Zimmermann, (1984) *J Anat* 138(2):351-63. [3]W.T. Coakley, D. Bazou, J. Morgan, G.A. Foster, C.W. Archer, K. Powell, K.A. Borthwick, C. Twomey, and J. Bishop (2004) *Colloids Surf B Biointerfaces* 34: 221-30.

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Pro-inflammatory Cytokines Inhibit Chondrogenesis by Human Mesenchymal Stem Cells through NF-κB-dependent Pathways

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INTRODUCTION: Cartilage loss through trauma or arthritis presents a major clinical challenge. The differentiation of mesenchymal stem cells (MSCs) into chondrocytes provides an attractive basis for cartilage regeneration. However, chondrogenesis will often need to occur in the presence of inflammatory mediators produced in response to injury or disease, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Among the intracellular mediators of IL-1/TNF activity are the nuclear factor-κB (NF-κB) family of transcription factors, which play important roles in the pathogenesis of osteoarthritis. Here we examined how these factors regulate the chondrogenic behavior of human bone marrow-derived MSCs.

METHODS: Vectors. Recombinant adenovirus encoding the cDNA for a 'super-repressor' inhibitor of NF-κB (srIκB) and driven by the CMV promoter [1] was provided by Dr. Paul Robbins (Pittsburgh, PA). Similar vectors encoding for green fluorescent protein (Ad.GFP) and firefly luciferase (Ad.CMV-Luc) were constructed previously [2]. Ad.NF-κB-Luc was acquired commerically.

hMSC Culture. MSCs were recovered from the intramedullary canal of patients undergoing hip hemiarthroplasty [3]. For chondrogenesis, passage-2 monolayers were transduced with Ad.srIκB or Ad.GFP. The next day, MSCs were formed into cell aggregates [4] and cultured with 10 ng/mL transforming growth factor-β1 (TGF-β1) and/or IL-1β or TNF-α (0-10 ng/mL each). After six weeks, aggregates were collected for biochemical analysis, histology, immunohistochemistry, and quantitative RT-PCR.

NF-κB Activity. hMSC monolayers were transduced with either Ad.NF-κB-Luc or Ad.CMV-Luc. The next day, cells were transduced again with Ad.srIκB or Ad.GFP. MSCs were then formed into aggregates and cultured with TGF-β1. After five days, a portion of aggregates were stimulated with IL-1β (10 ng/mL), and luciferase activities were measured after an additional five hours.

RESULTS: Both IL-1β and TNFα dose-dependently suppressed hMSC aggregate size and proteoglycan synthesis in response to TGF-β1. These effects were associated with a marked activation of NF-κB. Adenoviral delivery of srIκB dose-dependently blocked NF-κB-driven luciferase activity in response to IL-1β. Using this vector in the chondrogenesis model, we found that srIκB rescued proteoglycan (*Fig. 1*) and type II collagen synthesis within IL-1-stimulated aggregates. Although type X collagen followed this pattern, other markers of hypertrophic differentiation (ALP, MMP-13) responded differently.

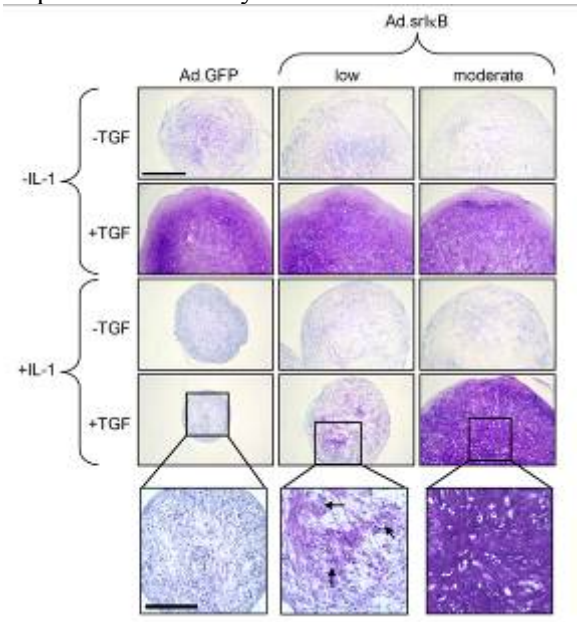

Figure 1: Toluidine Blue staining of 6-week hMSC aggregates. Scalebar = 0.5 mm. Magnified regions: scalebar = 0.2 mm.

CONCLUSIONS: Cell-based repair of lesions in articular cartilage will be compromised in inflamed joints. Strategies for enabling repair under these conditions include using specific antagonists against individual pyrogens, such as IL-1 and TNF, or the targeting of important intracellular mediators, such as NF-κB.

REFERENCES: 1. Iimuro *et al.* (1998) *J Clin Invest* 101(4): 802-11. 2. Palmer *et al.* (2003) *Methods Mol Biol* 215: 235-46. 3. Porter *et al.* (in press) *J Ortho Res* 4. Penick *et al.* (2005) *Biotechniques* 39(5): 687-91.

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Porous PVA-Chitosan Based Hydrogel as an Extracellular Matrix Scaffold for Cartilage Regeneration

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INTRODUCTION: Cartilage engineering benefits from the fabrication of random fibrous constructs, mimicking the structures found in the extracellular matrix of natural tissue, to support the attachment and proliferation of cells and ultimately convert the implanted cell-scaffold into native cartilage during cartilage repair.

METHODS: Preparation of hydrogels
PVA-117 (M_w = 74,000g/mol) was obtained from Kuraray Co. Ltd, Japan. Derivative chitosan (NOCC) was obtained from the Standards and Industrial Research Institute (SIRIM), Malaysia. The porous hydrogels were prepared from blends of PVA and NOCC in ratios (w/v) of 20-g PVA to 5% NOCC solution. The control PVA hydrogel was prepared in ratio of 20-g PVA to 100-ml of water. The polymer solutions were then cast into cylindrical molds and physically crosslinked by irradiation. The hydrogels were frozen prior to lyophilisation. Subsequently, the hydrogels were cut into discs approximately 2-mm in height, with a diameter of 5-mm.
Chondrocytes derived from New Zealand White rabbits were seeded at a density of $5X10^6$ cells on each pre-wetted scaffold and constructs cultured for 35 days prior to SEM.
The structure of the hydrogels was examined using scanning electron microscopy (SEM), followed by pore size measurement and swelling test. Data was compared and statistical significance was measured using parametric analyses ($p \leq 0.05$) and ANOVA.

RESULTS: SEM analysis revealed that PVA-chitosan based hydrogel contained an interconnecting porous structure ranging between 1-200µm in size unlike the PVA hydrogel which showed a non-porous structure with smooth and homogenous morphology. Penetration of chondrocytes and abundant extracellular matrix were observed in PVA-chitosan hydrogel constructs. Chondrocytes adhered to the scaffold covering large areas of the scaffold's surface, forming colonies while retaining their round morphology.

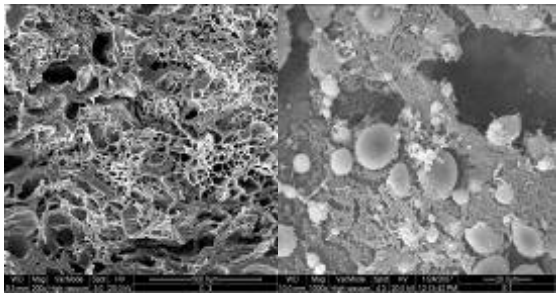

Fig. 1: The porous structure of PVA-chitosan hydrogel and cultured chondrocytes grow abundantly on the scaffold.

DISCUSSION & CONCLUSIONS: A novel porous PVA-chitosan based hydrogel has been developed which has the desired structure and pore size and enhances chondrogenesis of implanted cells. PVA-chitosan based hydrogel scaffold shows great potential as a cell carrier for cartilage tissue engineering. Swelling test will be relevant for biomechanical testing of the novel scaffold in future.

REFERENCES: [1] Griffon DJ, Reza-Sedighi M, Schaeffer DV, et al (2006) Chitosan scaffold: interconnective pore size and cartilage engineering Acta Biomaterialia **2**:313-320. [2]Hutmacher DW (2000) Scaffold in tissue engineering bone and cartilage Bomaterial **21**:2529-2543.

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Guiding Migration and Differentiation of Rat Bone Marrow Stromal Cells using d.c. Electric Fields *in vitro* – Implications in Bone Tissue Engineering

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INTRODUCTION: The application of d.c. electric fields (EF) for the regeneration of new bone *in vivo* has been reported by a number of groups [1,2]. The use of electric fields as a guidance cue to direct cell behavior in tissue engineering applications has been strongly focused on cardiac myocytes [3], although d.c. electric fields have been used to study the directed migration of bone cells from rabbits *in vitro* [4]; the majority of studies relating to bone or bone cells use electromagnetic fields. In this study we report the directed migration of rat bone marrow stromal cells in response to d.c. EFs, their directed migration on medical grade PLLA substrates, and initial evidence that the d.c. EFs can affect cell differentiation.

METHODS: Rat bone marrow stromal cells were obtained from 6-7 week old rats as described by Maniatopoulos et al [5]. The BMSCs were cultured either without or with osteogenic supplements during the experiments (10mM β-glycerophosphate and 10^{-8}M dexamethasone). Cells were allowed to attach to tissue culture plastic, or to PLLA substrates produced by compression molding, for 24 hours. A d.c. EF of 200mV/mm was then applied through the culture medium for 6 hours using an experimental set-up described elsewhere. Live time-lapse microscopy was used to monitor the cell migration. The ALP activity of BMSCs subjected to a d.c. EF of 20mV/mm for 2, 5 and 10 days was determined using p-nitrophenol measurements, and were normalized for total DNA.

RESULTS: In response to the applied d.c. EF, cells migrated towards the cathode. BMSCs cultured on tissue culture plastic surfaces in non-osteogenic medium migrated at a mean velocity of 7.9 μm/hr (sem = 1.0 μm/hr, n=61). In contrast, BMSCs cultured in osteogenic medium migrated at a significantly ($p < 0.0001$) greater velocity towards the cathode, with a mean velocity of 19.4 μm/hr (sem = 1.0 μm/hr, n=107). The differences in directed cell migration is demonstrated in Figures 1, where each red dot corresponds to the final location of an individual cell after being exposed to the EF for 6 hours, relative to a common starting point of the origin; the cathode is to the left of the images. BMSCs attached to the surface of PLLA substrates also migrated towards the cathode when exposed to an EF in osteogenic medium, but the velocity was only 7.7 μm/hr; the decrease in migration speed compared to results on tissue culture plastic may be related to differences in cell attachment.

ALP activity, normalized for total DNA, from BMSCs exposed to a d.c. EF (20mV/mm) showed a significant increase after 5 days of culture compared to no field controls.

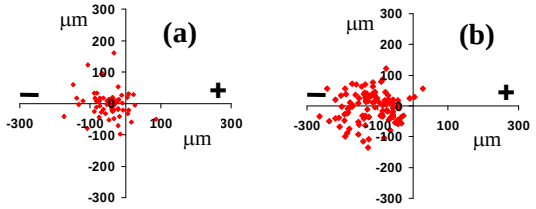

Fig. 1: Migration of BMSCs in a d.c. EF (200mV/mm) in (a) non-osteogenic and (b) osteogenic medium; cathode to the left.

DISCUSSION & CONCLUSIONS: The use of d.c. EFs (200mV/mm) can act as a strong guidance cue to the migration of BMSCs, although this response is affected by the cell phenotype and by the substrate surface chemistry. These results point towards d.c. EFs being a possible method for guiding cell migration through a porous scaffold.

REFERENCES: [1] 1 A.J. Meril (1994) *Spine*, **19**:2393-8. [2] A. Rogozinski, C. Rogozinski (1996) *Spine* **21**:2479-83. [3] M. Radisic, et al (2004) *Proc. Nat. Acad. Sci.* **101**:18129-34. [4] J. Ferrier, et al (1986) J. Cell Physiol. 129:283-8. [5] C. Maniatopoulos, J. Sodek, A.H. Melcher (1998) *Cell Tissue Res.* **254**:317-30

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HYDROXYAPATITE PARTICLES MAINTAIN PERI-IMPLANT BONE MANTEL IN OSTEOPOROTIC BONE

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INTRODUCTION: In osteoporotic bone, during remodelling of the initial woven bone peri-implant mantel to lamellar bone, resorption exceeds bone formation resulting in loss of implant osseointegration and loosening. Endobon® (Biomet) is a non-resorbable osteoconductive hydroxyapatite (HA) clinically used as bone filler. The goal of this study was to investigate if such a simple non-resorbable material could maintain a more dense and functional peri-implant bone structure, by shifting remodelling events in osteoporotic bone.

METHODS: Sixty 12 week old female Wistar rats were ovariectomized and 4 weeks later, osteopenia was verified with *in vivo* bone mineral density (BMD) measurements (XtremeCT, Scanco AG). Titanium screws were implanted bilaterally into the proximal tibial metaphysis. In the right tibia, the drill-hole was filled with 6 mg of HA particles before screw insertion. The rats were euthanized 1 hour, 2, 4, 6, and 8 weeks post implantation. Histomorphometric analysis was performed using Toluidine blue and Giemsa/Eosin staining protocols to differentiate between lamellar and woven bone in a 500µm thick peri-implant region of mid-sagittal sections. The amount of bone material and the contact rate, i.e. the percent of screw perimeter covered by bone tissue and HA particles, were assessed quantitatively at all time-points.

RESULTS: Ovariectomy successfully induced a mean decrease in trabecular BMD of approximately 30% after 4 weeks. Results from the histological analysis demonstrated that lamellar bone area percentage significantly increased with time in a similar way on both sides ($p=0.001$). After the same abundant increase in woven bone area during the first 2 weeks, woven bone was subsequently resorbed differently ($p=0.001$), whereby woven bone area percentage remained significantly higher on the HA-side compared to the control-side for the remaining experimental period ($p<0.001$) (Fig. 1). Besides the higher amount of bone material in the peri-implant region, HA particles induced more new bone in direct contact with the implant surface during the remodeling phase.

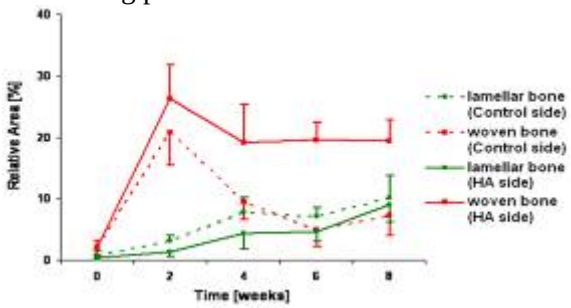

Fig. 1: Percent surface (mean ± SD) of lamellar (green) and woven (red) bone in the peri-implant region (—: HA side, ---: control side).

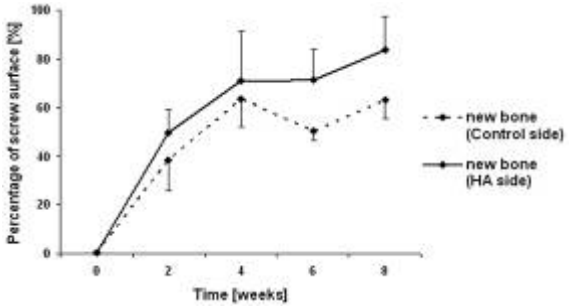

Fig 2: Contact rate (mean ± SD). Percentage of screw contour covered with newly formed bone (—: HA side, ---: control side).

DISCUSSION: The results of this study indicate that HA particles inhibit resorption of woven bone without affecting lamellar bone growth, thus resulting in maintenance of a denser peri-implant bone mantel in osteoporotic trabecular bone. Due to the presence of HA, remodelling processes were postponed and osseointegration was improved. This effect may play an important role in the prevention of implant loosening and cut-outs in clinical cases.

REFERENCES: [1] M. Yamazaki et al (1999), Oral Surg Oral Med Oral Pathol Oral Radiol Endod **87**:411-8. [2] K. Werber et al (2000), J. Hand Surg. [Am.] **25**:833-41.

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IN VITRO EVALUATION OF A NEW SYSTEM TO REDUCE PERI-IMPLANT STRAINS IN HORSES.

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INTRODUCTION: The use of conventional external fixators (EF) and of the transfixation pin (TP) casts in the treatment of distal limb fractures is often associated with complications in horses. The trickiest is the development of pin loosening, a painful process that exposes the horse to further and frequently fatal pathology like laminitis. The causative mechanism can be found in the considerable bending experienced by conventional pins resulting in pain and critical strains at the inner and outer cortex of a long bone depending on material properties and geometry of the EF components[1]. A novel pin-sleeve (PS) system was developed to decrease bone strains at the bone and EF pins interface. The goal of this experimental study was to model and compare the strain at the implant bone interface generated by the new system and the one generated by a commercially available transfixation pin when tested under axial compression in a bone substitute.

METHODS: Two canevasit hollow cylinders as diaphyseal bone models were instrumented with either the conventional (TP) or the new system (PS). The latter consisted of a 5-mm diameter pin running through an 8-mm diameter sleeve inserted into the canevasit. Pin and sleeve were in contact at two 1mm wide inner sleeve supports located at the centre of each cortex. The 6.3-mm diameter, positive and centrally threaded TP was applied in a standard manner. Uniaxial 120Ω strain gages (SGs) were glued on the external surface of the hollow cylinders centered above the implants. The outer support for both systems was a resin fiber 10 mm thick cast. Four different configurations of the new PS system were tested, applying an axial preload of 3, 4.5, 5.5 and 6kN to the threaded pin using nuts and maintained via a ring incorporated into the cast. The lower end of the cast was embedded in PMMA and fixed to the table of a material testing machine. The bone substitutes were loaded in axial compression (range: 50N-2500N) at 1 Hz for 2000 cycles. Each configuration was tested three times. Axial cylinder displacement and strains were recorded and compared. Finite element models (FEM) of both instrumentation methods were developed and validated comparing strains and displacements between the experimental and the computational model. Correlation coefficients were computed between preloading and axial displacement and strains. FEMs were used to describe the distribution of strains.. Significance level of statistical test was set at $p<0.05$.

RESULTS: The PS system had always comparable axial displacement with a substantial decrease in strains as compared to the TP (*Table 1*). Preloading was found to be inversely correlated to the axial displacement (r_P=-0.93; $p<0.01$) but did not correlate significantly with the measured strain (r_P=-0.33; p=0.29). The FEMs were validated having maximum error of 14% between the experimental and FEM strain and displacement values. Based on these models (*Figure 1*) the maximum strains 2mm above the implants were 5500µstrain in the TP and 500µstrain in the PS system.

EXPERIMENTAL	Displacement (mm)	µstrain
Transfixation pin	0.99 ± 0.06	2841 ± 30
Pin-sleeve 3kN preload	1.09 ± 0.03	467 ± 2
Pin-sleeve 4.5kN preload	0.96 ± 0.02	463 ± 8
Pin-sleeve 5.5kN preload	0.92 ± 0.01	473 ± 7
Pin-sleeve 6kN preload	0.87 ± 0.02	467 ± 7

Table 1: Mechanical testing results (mean ± SD).

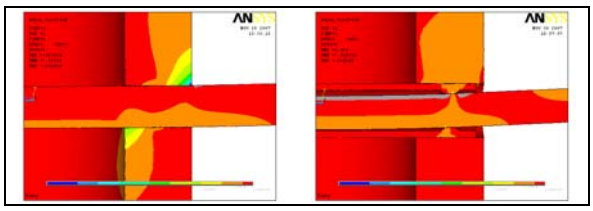

Figure 1: Axial strain distribution in one cortex for the two systems (range:-25000÷1000µstrain).

DISCUSSION & CONCLUSIONS: This study demonstrated that the novel system has the potential to reduce the risk of pin loosening and improve the clinical performance of external fixators. Further studies are required to investigate ultimate load wear and fatigue properties.

REFERENCES: [1]Huiskes,R., Chao,E.Y., and Crippen,T.E. (1985) Parametric analyses of pin-bone stresses in external fracture fixation devices. J.Orthop.Res., 3:341-349.

CORROSION AND TISSUE REACTION TO THREE GUIDE WIRES (MP35N, L605 & 316L) IN COMBINATION WITH A CONVENTIONAL 316L STAINLESS STEEL CANNULATED SCREW

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INTRODUCTION: Cannulated screws, along with guide wires, are commonly used for a variety of orthopaedic indications, but typically for fractures in cancellous regions. Breakage or bending deformation of the guide wire is a clinical concern. Therefore it would be advantageous to use Co-Cr alloys such as MP35N and L605 in these applications as the occurrence of breakages would be reduced. However, if the Co-Cr alloy should break in situ, galvanic or crevice corrosion cannot be ruled out. Therefore, we designed an experiment to determine if galvanic or crevice corrosion occurred in the *in vivo* environment. Implant devices were designed to replicate a clinical situation where dissimilar metals could potentially form a galvanic couple.

METHODS: Custom-made implants were developed (fig 1). In all cases the screw was 316L stainless steel while the guide wire (centre component in fig 1) was interchanged between 316L (control), MP35N and L605. The cannulated part of the screw had a conical shape. This construct was considered to imitate a cannulated screw with a broken guide wire remaining in situ.

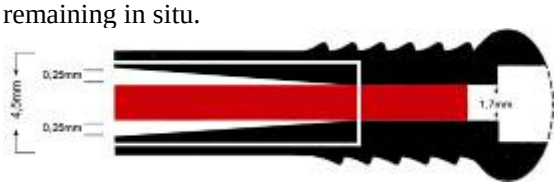

Fig 1: *The design of the implants to test crevice and/or galvanic corrosion.*

Samples were implanted into the distal femur and proximal tibia of Swiss mountain sheep for both 1 and 6 months. Post mortem samples were analysed using Scanning Electron Microscopy (SEM; n=8 per group) and histological analysis (n = 8 per group). Both SEM and histological analysis was performed at multiple areas along the length of the screw to determine if the crevice size had an effect on the cellular infiltration and corrosion.

RESULTS: From SEM analysis of the screws and guide wires, no visible signs of corrosion were observed at either time point or for either group. However, there were mechanical manufacturing marks and tissue residues after enzymatic cleaning were visible on all samples.

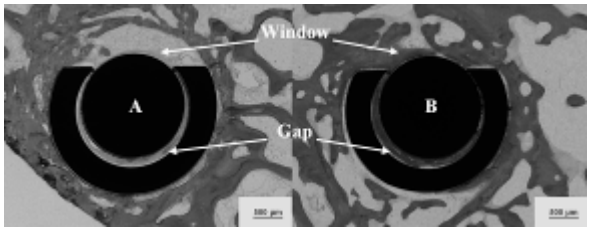

Fig 2: *A) 316L guide wire after 1 month implantation showing limited bone re-growth into the drill hole created for placement of the implant; B) L605 Co-Cr alloy guide wire after 6 month implantation showing bone formation in front of the window and in the gap*

DISCUSSIONS & CONCLUSIONS: In the literature the results of coupling Co-Cr alloys and stainless steel is inconsistent. Younkin states that MP35N is extremely noble and caused galvanic corrosion of 316L and carbon steel in seawater tests. However, Reclaru et al. evaluated the galvanic current of a Co-Cr/REX 734 steel couple, and concluded that there was no appreciable risk for a crevice corrosion caused or amplified by the galvanic coupling. In this work, no major signs of corrosion were visible using SEM. However, some localised artefacts were observed that could have indicated corrosion. The incidence of artefacts was sporadic and did not vary considerably between test groups.

Particles observed on histological evaluation could potentially be of concern; however, the fact that no significant difference was detected between groups, indicates that the use of Co-Cr alloys as guide wires would not increase the presence of particle formation in vivo. Furthermore the absence of an associated cellular reaction around the particles might support the possibility that these particles were created as an artefact of either the model used, or the analysis methods.

Despite the comprehensive evaluation of tissue from the animals in this study, we could not detect an adverse *in vivo* effect of using dissimilar materials compared with stainless steel alone in a model of a broken guide wire in a cannulated screw.

REFERENCES: Reclaru L, et al. Biomaterials 2002; 23: 3479-85.

Younkin CN. J Biomed Mater Res Symposium. 1974;5:219-26.

An *In Vivo* Implantation Study in New Zealand White Rabbits for Granular Hydroxyapatite

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Introduction: We developed hydroxyapatite (HA) granules made from local raw materials (GranuMaS™), which have been fabricated using a novel method (Patent (Pending) No. PI 2004 0748). GranuMaS™ have been fully characterized using X-ray diffraction, scanning electron microscopy, energy dispersive x-ray, Fourier transform infra-red spectroscopy and inductively coupled plasma methods. It has also been shown to comply with the ASTM F1185 - 88 (1993) specification standards.
Biocompatibility studies have been performed on the product, which include neutral red cytotoxicity assay using cultured fibroblast and CRL-1427 osteoblast cells; MTT (Tetrazolium Salt) assay; cytotoxicity studies for apoptosis using Acridine Orange / Propidium Iodide (AO/PI) dual staining of V79 cells in DMEM; and COMET assay genotoxicity studies using L-929 (normal mouse epithelial) cells.
An *in vivo* implantation study involving New Zealand White rabbits were carried out in the course of this study.

Materials and Methods: HA granules of between 250 to 500 µm in diameter were implanted into a 9mm by 4.5mm defect made in the proximal metaphyseal region of the rabbits' left tibia. The tibia was harvested at 2, 3, 4, 6 and 12 weeks. The retrieved specimens were processed to produce undecalcified tissue sections using a hard tissue band cutting and microgrinding system (EXAKT Apparatebau, Germany). Prepared slides were analyzed under the light microscope using compound polarized light, Toulidine Blue, Masson Goldner's Trichrome and von Kossa stains.

Results and Discussion: New bone formation is seen even at 2 weeks post-implantation, which further consolidates at 3, 4 and 6 weeks. At 12 weeks the new bone between the granules are well-formed and the cortical defects made have closed off with well-formed lamellar bone seen between the granules. There is direct apposition of the new bone to the granules. The new bone seen with bridging of the medullary cavity and no major voids or defects found. No evidence of any fibrous or inflammatory tissue was seen.

Conclusions: GranuMaS™ is osteoconductive and biocompatible when implanted in rabbits. It could be used as an alternative bone graft substitute, as there is profound new bone formation in-between and around the granules.

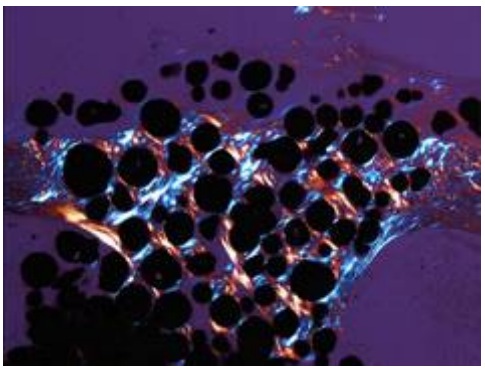

Figure 1: 12 weeks post implantation seen under compound polarized light (magnification 2.5x)

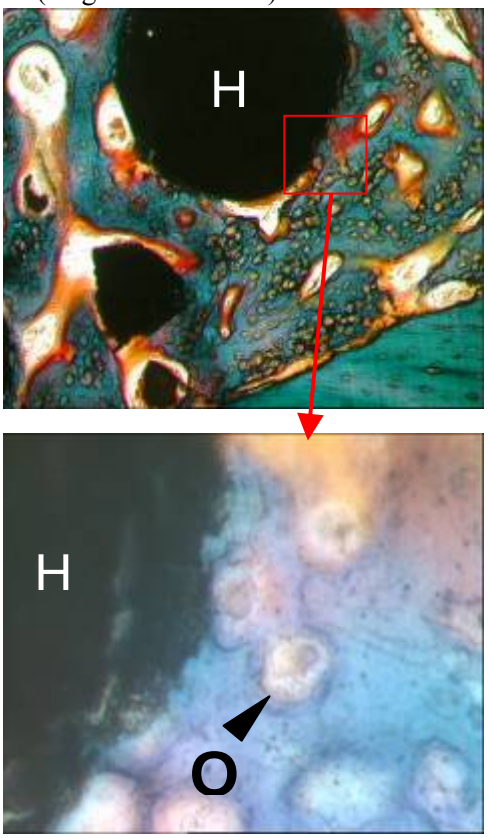

Figure 2: Masson Goldner's Trichrome at 3 weeks of implantation.
(HA – hydroxyapatite, O –osteocytes at 10X and 100X magnification)

GRANULES HYDROXYAPATITE APPLICATION IN FRACTURES

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INTRODUCTION: In conjunction with a multi-institution research project, towards achieving the production of a locally made bone graft substitute. Hydroxyapatite (HA) granules made from local raw materials have been fabricated using a novel method (Patent (Pending) No. PI 2004 0748). The granules were characterised both chemically and physically and found to abide by the ASTM F1185 - 88 (1993) specification that covers the material requirements for HA intended for use as surgical implants. A phase 1 clinical trial involving the use of the granules for root socket obliteration in young healthy adults following tooth extraction was performed. Based on the results of these studies, the material was approved for a phase 2 clinical trial. The study was approved by the University and Hospital Clinical Ethical Committee in July 2005

Objectives: 1) To study the usage of GranuMas™ as bone substitute material.
2) To analyse the common conditions patterns that need bone substitute material.
3) To study the functional outcome of operative treatment after the usage of GranuMas™.

METHODS: Young healthy adults (age above 18) who have had recent trauma resulting in a closed fracture that requires bone grafting with no other complications were included in the study. Non union which is uninfected was included in the case study. Most of the patient was involved in road traffic accident.
Inclusion and exclusion criteria were used to guide the selection.Clinical and radiological observations were made and recorded. Radiograph was taken at post-operatively, 6, 12 weeks, 6 months and yearly basis.
Measurements are made and the results was analysed. This represents the results of patients that have been operated on since August 2005

RESULTS: 35 patients were treated using the GranuMas™ as bone substitute. All showed excellent and good results. No collapse of bony part noted during follow up. Union was noted in all of the cases. The granules degradation was slow this is noted if large amount of HA being used. All patients recover well post operatively.Cases operated includes distal end of radius fractures, non union femur, comminuted fractures head of radius, supracondylar femur and tibial plateau fractures. Locking plates (AO plates) was used in most of the cases as implant.

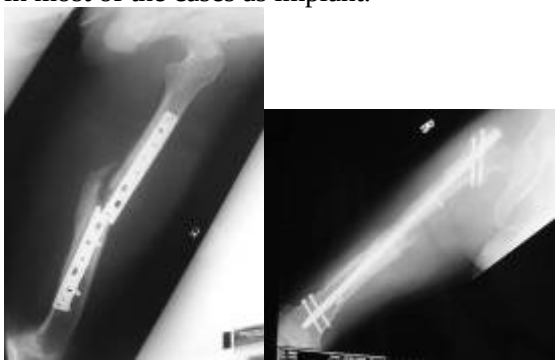

Figure 1: Non union and at 1 year post nailing

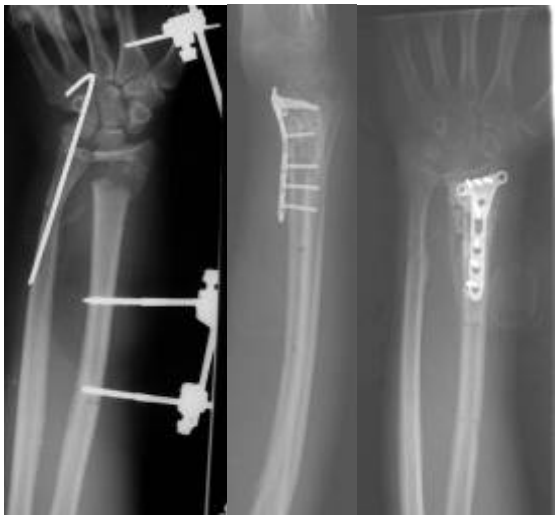

Figure 2: Fracture distal radius with bone loss and 6 months post plating

DISCUSSION & CONCLUSIONS:
Union noted in all cases. The HA granules showed osteoconduction functions as for the bone to heal. Callus formation noted in all cases. GranuMas™ usage showed good outcome results as bone substitute material.

SURFACE POLISHING POSITIVELY INFLUENCES EASE OF FRACTURE FIXATION PLATE & SCREW REMOVAL, & THE SURGICAL TIME REQUIRED FOR EXTRACTION.

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INTRODUCTION: Difficulty to remove fixation devices due to excessive bony on-growth results in extended surgical time which can lead to excessive blood loss, debris contamination, and other potential risks associated with superfluous surgery. Commercially available locking compression plates (LCP) and screws are manufactured for clinics with a standard, micro-rough surface. However, it is this surface, which we believe contributes to the excessive bony on-growth reported. We have recently shown that by reducing the surface micro-topography of commercially available materials via surface polishing, that the removal torque required for intramedullary nails can be reduced. In this study, we have applied this technology to LCP constructs, to assess if surface polishing can alleviate problems with excessive bony overgrowth, in a locked plate system.

METHODS: Materials are outlined in fig1. The surface topography of each material was characterised using non-contact white-light profilometry, SEM, and contact angle while the surface chemical composition was analysed using XPS. Approval to perform the *in vivo* part of this study was granted by the Cantonal Animal Ethics Committee (GR #6/2006). Eighteen Swiss Alpine sheep were implanted with two test constructs on each tibia, in a bilateral (all 4 constructs were implanted within the same sheep), non-fracture model. Sheep were separated into 3 groups according to the timepoints for euthanasia; (6, 12 &18 months).

RESULTS: Electropolished (EP) TAN (NE) screws (p=0.001), paste polished (PP) TAN (NP) screws (p=0.01) and stainless steel (Ss) screws (P=0.000) were significantly easier to remove compared to standard micro-rough (Std) TAN (NS) screws. Similarly, PP significantly (p=0.000) influenced the quantity of bone contact, compared to NS. However, compared to NS screws, NE screws were not found to have significant differences in bone contact (p=0.066).

DISCUSSION&CONCLUSIONS: After explantation it was observed that soft tissue was present in the combination holes of the EP, PP and Ss constructs. This tissue was easily removed with a scalpel and K-wire, and generally took less than 5 minutes per construct. For the majority of Std systems, bone overgrowth on the plates, and in-growth into the combination holes was noted. This bone became increasingly difficult to remove with time, and dramatically increased the operational time (4 times that for polished systems) for removal. The significant reduction in time required for tissue removal from polished devices, will directly reduce the surgical time associated with implant removal, thus improving, not only the economic burden associated with surgical procedures, but also the surgical related complications with regards to the patient, which are both principal deciding factors for implant removal.

Fig.1. Constructs used included Std cpTi LCP/TAN screws; EP cpTi LCP/ TAN screws; PP cpTi LCP/ TAN screws; Ss LCP/ screws.

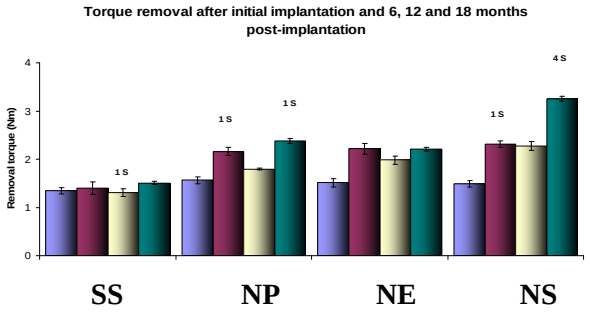

Fig2. Screw removal torque after initial insertion (blue), 6 (Red), 12 (yellow) and 18 (green) months implantation.

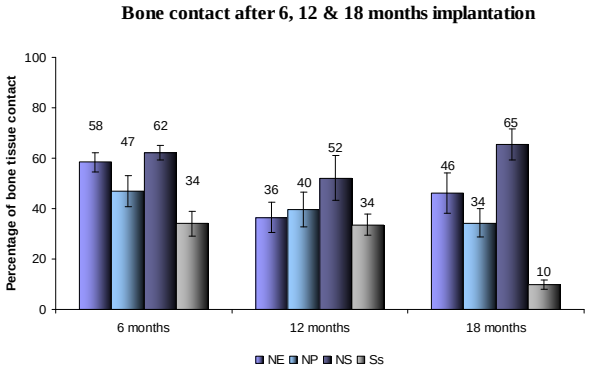

Fig.3. Percentage of bone contact for (left to right) NE, NP, NS, SS screws after 6, 12 and 18 month implantation.

Surface polishing eases intramedullary nail removal – A novel *in vivo* study

JS Hayes[1], DI Vos[2],J Hahn[1] SG Pearce[1], RG Richards[1]

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INTRODUCTION: Intramedullary (IM) nails are fabricated for orthopaedic clinics from either titanium-6%aluminium-7%niobium (TAN) or electropolished stainless steel (SS). Yet, removal of TAN IM nails often produces more extraction related complications compared Ss IM nails of the same design. Despite the knowledge that surface microtopography can be a major determinant of osseointegration, this avenue, in terms of IM nailing technology, has not previously been explored as a potential resolution to issues involving device removal. In fact, many studies suggest alternative methods for removal only once conventional methods have failed. In this study, we present a novel, and simple method for reducing the problems associated with IM nail removal, and resulting intra-operative complications, due to excessive bony on-growth.

METHODS: Study approval was granted by the Cantonal animal ethics committee (GR 5/2006). Commercially available Synthes® 9.5mm Universal Humeral Nails (UHN) made of shot-peened electropolished stainless steel (SS) titanium-6%aluminium-7%niobium (TAN) with either a standard micro-rough surface (S-TAN) or experimental smooth paste-polished surface (PP-TAN) were studied. Fourteen adult female Swiss Alpine sheep were divided into two groups of 7. Using a bilateral, non-fracture model, seven sheep were implanted with S-TAN nail in one tibia and a SS nail in the contralateral tibia. The remaining 7 sheep were implanted with an S-TAN nail in one tibia and a PP-TAN nail in the opposite tibia. After 12 months implantation, mechanical pull out tests were performed on 6 of the 7 nails from each group ($p<0.05$). The remaining nail stayed *in situ* for histomorphometric analyses.

RESULTS: Pullout tests demonstrated that the SS nails had a significantly lower pullout force than S-TAN nails implanted in the same sheep ($p=0.028$; Fig1A). Removal of PP-TAN nails demonstrated a markedly lower ($p=0.043$) pullout force in comparison with the S-TAN nails implanted (Fig1B). Observations after removal showed bone growth directly within the nail PP-TAN interlocking holes however any tissue within the screw holes was easily displaced with a K wire (Fig. 2A). Contrastingly, within holes of S-TAN nails, bone in-growth was evident (Fig. 2B). Furthermore, this hard tissue could not be displaced.

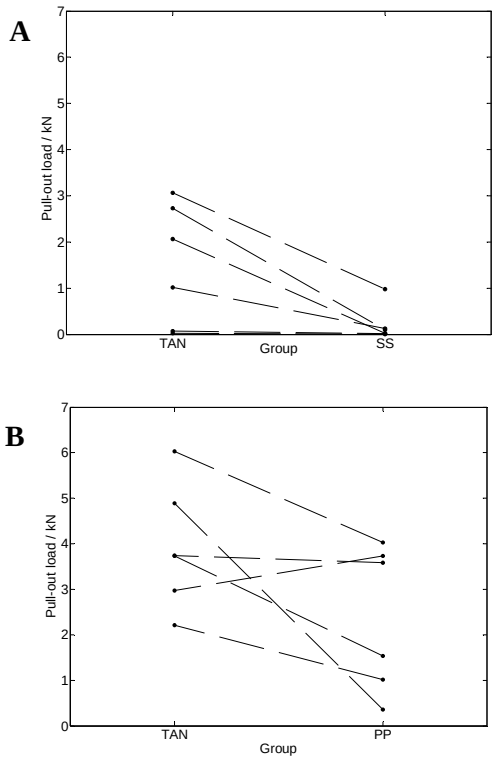

Fig.1. A significant reduction in extraction force was observed for SS ($p=0.028$) compared to S-TAN IM nails (Fig1A) and PP-TAN ($p=0.043$) compared to S-TAN IM nails (Fig1B).

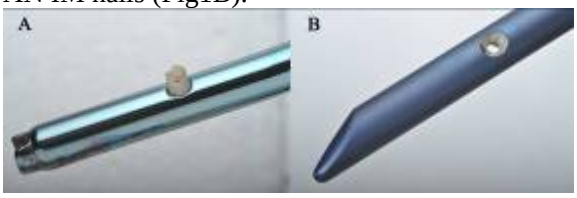

Fig.2. Tissue within interlocking holes of PP-TAN nails was easily displaced with a K wire (Fig2A) but S-TAN nails promoted in-growth of bone and this could not be removed (Fig2B).

CONCLUSIONS: Since TAN is preferred over EPSS for IM nailing due to its better biocompatibility and mechanical properties, we believe these findings will be used to recommend changes to current surface technologies of intramedullary nails, to reduce complications seen with nail removal, especially in rapidly growing bone in paediatrics.

ACKNOWLEDGEMENTS: A.Schlienger (Synthes, CH) for the implants; A. Pearce for initial planning; K. Schwieger for statistical analyses; B.Gueorguiev for pullout tests; (all AO Research Institute) and the animal care staff from the AO Research institute.

PHOTO-CROSSLINKING COLLAGEN GEL FOR TISSUE ENGINEERED CARTILAGE

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INTRODUCTION: Numerous hydrogels have been developed as three-dimensional scaffolds for chondrogenesis. Solubilized collagen is an ideal natural material that can be used as a scaffold because of its biocompatibility and biodegradability. Although spontaneously forming collagen gels can conform to a cartilage defect, they are soft and unstable. Kochevar et al. reported that type I collagen can be crosslinked using photoreactive dyes, such as riboflavin (vitamin B2), and exposure to visible light. *In situ* gel crosslinking could induce molecular interactions with the native cartilage surrounding the lesions to stabilize the gel during cartilage formation. The governing hypotheses of this work are: 1) photochemical crosslinking can be used to generate stable collagen hydrogels; 2) Chondrocytes encapsulated in the hydrogels form neocartilage; 3) the neocartilage will integrate with existing cartilage.

METHODS: Chondrocytes were suspended in 4 test concentrations of riboflavin solution (0.1–1 mM). The cell suspension was mixed with an equal volume of 0.5% type I collagen solution. The suspension with a final cell concentration of 40×10^6 cells/ml was poured onto 6 well culture plates and photocrosslinked using 4 irradiation test doses of visible light. Control samples were not subjected to irradiation. The constructs were cultured up to 10 days to evaluate cell survival with live-dead assay.

Implantation of photocrosslinked constructs (n=8) was performed to determine whether this novel method would allow the construct to make hyaline cartilage in the *in vivo* environment. Specimens were evaluated histologically (Safranin-O and immunohisto-chemically for COL 1 and COL 2) and biochemically (collagen and GAG content). To evaluate the ability of the gel to permit cartilage formation and integration with the surrounding native cartilage, photocrosslinked gels with cells were placed between discs of knee cartilage and implanted in mice.

RESULTS: Cell viability remained high with short irradiation times at all concentrations of riboflavin. Increased irradiation and riboflavin concentration had a negative effect on cell viability (Fig 1). Specimens placed in mice showed neocartilage formation as evidenced on specimens stained with Toluidine blue (Fig 2a) and Safranin-O and produced GAG (Fig 2b) and type 2 collagen (Fig 2c) Neocartilage between cartilage discs formed tight bonds with existing cartilage (Fig 2d).

Figure 1

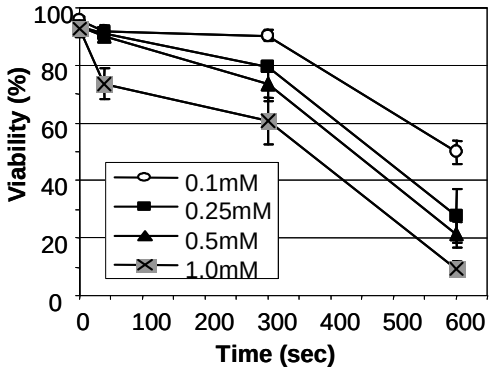
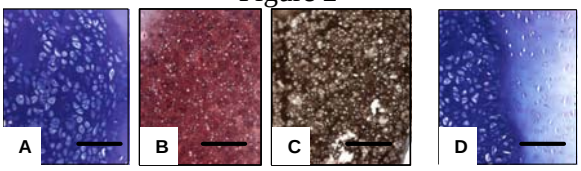


Figure 2



DISCUSSION & CONCLUSIONS: Crosslinking collagen into hydrogels can be achieved using benign light sensitive photreactive dyes like riboflavin and visible light. Photchemically crosslinking the collagen solutions containing chondrocytes permits cell survival and neocartilage formation. As such, collagen containing chondrocytes could be injected into a defect site and polymerized in situ. The crosslinking process could stabilize the hydrogel in the defect and permit new cartilage formation to restore the joint surface. The results from this study encourages further study in large animal joint models.

ACKNOWLEDGEMENTS: These studies were supported in part by the AO foundation.

MONITORING OF CELL MIGRATION

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INTRODUCTION: Cells contacting an implant are affected by the implant surface. Latter interaction is an important key feature that determines the clinical success of implants. Beside the state of differentiation also the parameters cell shape, cell orientation, migration direction and migration velocity may be affected. Especially the latter two parameters (migration direction and migration velocity) may have consequences for the coverage of the implant by these cells. The monitoring of cell migration and its analysis are of key importance in order to elucidate the mechanisms on which (directed) cell migration is based.

METHODS: Cells (3T3 fibroblasts) were labelled before starting the migration experiments with a fluorescent dye (DiI). The structured surfaces with the labelled cells were placed in a special incubation chamber with a cover glass lid. This chamber allowed the online observations of the migrating cells with a confocal laser scanning microscope (CLSM) under cell culture conditions. Cell migration was monitored for several days by taking a picture each 15 minutes from the same previously selected areas of interest. Based on the obtained sequence of pictures of each cell, the migration pathway (trajectory), cell shape, migration direction and migration velocity were calculated by special image analysis software. Cell migration was analysed on a titanium alloy sample with a plane surface and 10 different micromaschined structures (grooves/ridges). The width of the ridges and grooves as well as the inter ridge/groove distance was in the range of 5 to 40 µm. The structured surfaces can be divided in V-shaped surfaces, U-shaped surfaces and ∩–shaped surfaces. Cell migration was monitored on all types of surfaces at the same time [1, 2].

RESULTS and DISCUSSION: The fibroblast cells were strongly affected by the surface structures and the extent was structure dimension dependently. The frequency of cells with a circular shape was higher on the plane surface, than the amount of cells with circular shape found on the structured surfaces. No significant difference in the frequencies of a certain type of cell shape was found among the different structured surfaces. On structured surfaces the fibroblasts significantly preferred orientating themselves parallel to the axis of the grooves/ridges within a sector ± 10°. Among all structured surfaces, structures with shallow grooves/ridges (5 µm width) and large plane sections between the grooves/ridges exhibited the lowest number of cells orientated parallel to the structures. Cells migrating on the plane surface showed a migration direction respectively migration angle, which was evenly distributed in all directions. Cells, which were seeded on the structured surfaces, migrated preferentially parallel to the grooves/ridges. Switching of tracks was depending on the dimensions of the grooves/ridges but also on the width of the tracks. On larger tracks the cells did not have the tendency to switch the tracks as often as they do, when they migrate on narrow tracks. On the various structured surfaces different mean migration velocities were observed. 3T3 fibroblasts cells migrating on the plane surface were by far not migrating with the lowest velocity. Cells migrating on structures with large plane tracks between the grooves, respectively large flat areas between the ridges exhibited the highest mean migration velocity. Structuring of the surface resulted in an increase in cell migration velocity parallel to the grooves/ridges compared to the overall mean velocity on that certain surface.

REFERENCES: [1] J.-P. Kaiser, A. Reinmann and A. Bruinink (2006) The effect of topographic characteristics on cell migration velocity, *Biomaterials* **27**: 5230-5241.

[2] J.-P. Kaiser and A. Bruinink (2004) Investigating cell – material interactions by monitoring and analysing cell migration, *Journal of Materials Science: Materials in Medicine **15**:* 429-435.

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The effects of oral glucosamine sulphate and chondroitin sulphate on focal (traumatic) cartilage damage

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INTRODUCTION: It is a well established fact that focal cartilage damage is difficult to treat owing to its poor reparative ability. Treatment options are generally limited to surgical modalities and although oral supplements using glucosamine sulphate and chondroitin sulphate have been advocated for osteoarthiritis, these medications have not been used for the treatment of focal cartilage damage. To establish the effects of these supplements on focal cartilage damage, an experimental trial was performed using rabbits.

METHODS: 18 New Zealand white rabbits weighting approximately 2.5kg and 6 months old were used in this experiment. Animal experiments were conducted in accordance to the conditions approved by the ethical committee on animal experimentations in University of Malaya and in accordance to Malaysian laws and regulation on animal experimentations. Rabbits were anaesthetized accordingly. A focal full thickness articular cartilage defect measuring 5mm in diameter and 3 mm in depth were created on the medial condyle. After 2 weeks following the surgery, oral glucosamine sulphate (GS) and/or chondroitin sulphate (CS) were gavaged on a daily or weekly basis as according to the drug manufacturers' instructions. The dosages of medication provided were appropriate to their body weight as described in previous literatures. The rabbits were divided into 3 groups with each group consisting of 6 rabbits: The control Group I (n=6), Group II treated with only GS (n=6) and Group III treated with GS and CS. Three rabbits in each group were euthanized at the end of 3 and 6 months and the operated site were sent for histological, immunohistology and quanitfication analyses.

RESULTS: Although a general histological view of the sites revealed slightly improved repair quality in Group III as compared to the other groups, based on the Brittberg histological scoring system, there were no differences between these groups. However, we found that there were notable differences between the concentration of S-GAG expressed between groups II and III as compared to group I. Differences were only noted at the end of 6 months and not at the end of 3 months.

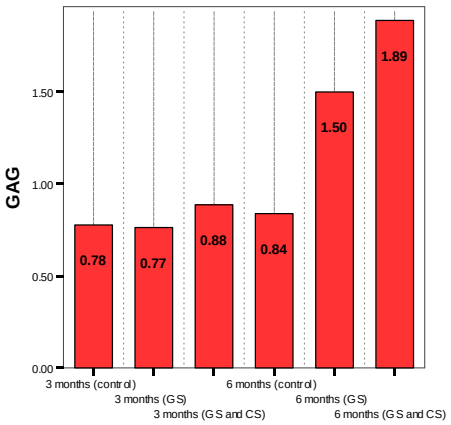

Fig. 2: Comparison of the amount of S-GAG in the defect sites of the different groups (GS=Glucosamine Sulphate; CS=Chondrotin Sulphate)

DISCUSSION & CONCLUSIONS: From this preliminary study we can suggest that GS and CS may play a role in the treatment of traumatic focal cartilage damage however from our statistical analyses; these results were not significant owing to the small sample size. Future studies involving a larger number of subjects may be required before the use of GS and CS can be justified for patients with traumatic cartilage damage.

REFERENCES: [1] Beth J.C., Uhl T (2001) *J Athletic train* **36(4)**:413-9. [2] Derfoul A., Miyoshi A.D., Freeman D.E. *et al* (2007) *Osteoarthritis Cartilage* **15**:646-655 [3] Chan P.S., Caron J.P., Rossa J.M. (2005) *Osteoarthritis Cartilage* **13**:387-394

ACKNOWLEDGEMENTS: This research was funded by Ministry of Science and Technology Malaysia under the 8th Malaysian Plan (36-02-03-6037). Glucosamine sulphate was provided by courtesy of Rotta Pharmaceuticals (Malaysia).

Tenocyte alignment is dependant upon cell density and tensional loading

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INTRODUCTION: Although it has been accepted that tenocyte responds to mechanical stimulation, (following a process known as mechanotransduction) little is known of its physical response when tensional force is applied through them. Although there have been some reports of cell behaviour in response to tensional loading, these studies have not taken into account *In Vivo* conditions including cell to cell contact, which is major influencing factor. Our study aims to prove that cell contact and different applied strains affect cell behaviour.

METHODS: Primary cell cultures were performed using remnants of biceps tendon tissues from patients undergoing shoulder reconstruction. Cells were passaged to P2 before being seeded onto our custom made collagen type I coated silicone flasks. Different seeding densities were cultured in low-glucose DMEM with 10% foetal calf serum (FCS). Cells were left to grow in these flasks for 4 days prior to loading. These flasks were placed on our custom made motorised tensional loading jig within our culture incubator. While one end is fixed in a static clamp, the flasks are subjected to cyclic unidirectional loading by the motor. Loading strains of approximately 3% and 6% at 1 Hz cyclical loading patterns were applied. Observations were made with photographs of cultured cells taken at specific placement points within the flasks at 1, 3, 6, 24 and 48 hours. Image analyses were performed accordingly.

RESULTS: It was observed that at 6% strain, cells at higher densities showed different alignment patterns than that of lower cell densities (where there are lower or absent cell to cell contacts) favouring an alignment pattern in parallel with the direction of pull. These changes can be seen as early as 6 hours from the start of loading. In contrast, cells which had minimal contact with other surrounding cells appear to be aligned perpendicular to the direction of pull. However, at 3% strain (or less), cells at higher densities appear to align more perpendicular to the direction of loading. At similar strain levels, cells which are not in contact with each other appear to have little or no response to loading. In addition, they appear to have more rounded morphology at the end of 24 hours.

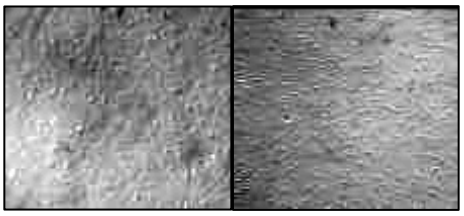

Fig. 1: Pictures taken prior to loading at 6% strain (left) as compared to pictures taken at the end of 24 hours (right) of cells at high densities. Note that the cells are aligned towards the direction of loading

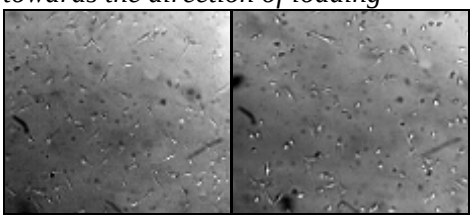

Fig.2: Pictures taken prior to loading at <3% strain (left) as compared to pictures taken at the end of 24 hours (right) of cells at low densities. Note the loss of normal tenocyte morphology.

DISCUSSION & CONCLUSIONS: Our experiments demonstrate that cell seeding density and level of strain are important factors to consider when conducting experiments simulating *In Vivo* conditions. In comparing our experimental model against histological pictures of whole tendon sections, similar cell morphologies were found. Thus this experimental model replicates *In Vivo* conditions.

REFERENCES: [1]M.Eastwood, D.A. McGrouther, R. A. Brown (1998) *Proc Instn Mech Engrs* 212(H):85-92 [2] C. Neidlinger-Wilke et al (2001) *J Orth Res* 19:286-293 [3] W.A. Loesberg et al (2005) *J Biomed Mater Res A.* 1;75(3):723-32

OPEN-POROUS CERAMICS FOR BIO-APPLICATIONS

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ABSTRACT: There are many different approaches how to produce porous ceramics containing more than 60 vol% of air: i.e. the replica method, the sacrificial template method and the direct foaming of liquid suspensions [1]. The direct foaming method is of particular interest due to its simplicity, low cost process, versatility and potential for in-situ applications.

We present here a novel direct foaming method which produces foams that are stable against bubble coarsening and drainage in the wet state. This stability is reached through the irreversible adsorption of particles onto the air-water interface. Upon drying and heat treatment or introducing of a setting agent, porous ceramic bulk material can be produced [2].

The aim of this work is to elaborate a tool to further control the microstructure of these porous ceramics, namely the open porosity and the pore size. The results show that upon combining a controlled destabilization of the wet foams with a non-toxic setting reaction such as a calcium aluminate cement reaction, the pore size and the open porosity can be tailored within a wide range, featuring porosities of 75 to 95 vol% air and pore sizes between 75 µm and 1 mm. Preliminary results with sintered (heat treated) calcium alumina samples demonstrate the potential of these foams to host cells of different types. We have observed that human osteosarcoma and endothelial cells attached to the material after 24 hours of incubation, proliferated for up to 4 days and colonized the entire cross-section of the ceramic. The non-toxicity of these foams was also evidenced.

Further work is now being done towards processing of calcium phosphate materials and in a later step to possibly produce injectable open-porous bone cements.

REFERENCES:

1. Studart, A.R., et al., *Processing routes to macroporous ceramics: A review.* Journal of the American Ceramic Society, 2006. 89(6): p. 1771-1789.

2. Gonzenbach, U.T., et al., *Ultrastable particle-stabilized foams.* Angewandte Chemie-International Edition, 2006. 45(21): p. 3526-3530.

CHONDROGENESIS OF BONE MARROW AND PERIPHERAL BLOOD DERIVED ADULT HUMAN MESENCHYMAL STEM CELLS

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INTRODUCTION: Although mesenchymal stem cells (MSCs) isolation from a number of tissue sources have been described, very few literatures have reported successful isolation of adult MSCs from peripheral blood or its chondrogenic differentiation for clinical applications. The objective of this study is to isolate MSCs derived from both human bone marrow and peripheral blood and to compare their potential to undergo chondrogenesis. **METHODS:** Bone marrow (BM) and peripheral blood (PB) samples were collected into blood collection tubes containing lithium heparin (2-4mls). Mononuclear cells were separated from both types of samples using Ficoll–Paque PLUS by centrifugation and suspended in cell culture medium before being plated onto tissue culture flasks. Suspended cells were subsequently removed after 5 days of culture, and adherent cells were left to grow. Cells were sub-cultured (2–5 passages) prior to further cellular analyses and differentiation experiments. Chondrogenic pellets were harvested after 4-5 weeks in culture. To assess chondrogenesis, alcian blue and safranin-O were used to determine whether cartilage matrix proteoglycan was expressed. Chondrogenesis was also quantified by the amount of sulphated glycosaminoglycan (S-GAG) production measured using 1,9-dimethylmethylene blue (DMMB) assay.

RESULTS: Based on our results, we were able to establish techniques for isolation of MSCs from BM and PB. The presence of surface marker proteins CD44, CD105, CD166 and the absence of CD34 in these cells (confirmed using flow-cytometry) indicate the high likelihood of successful mesenchymal stem cell isolation. Histological examination revealed significant cellular expressions of proteoglycans and glycosaminoglycans indicating successful induction of chondrogenesis in our isolated MSCs.

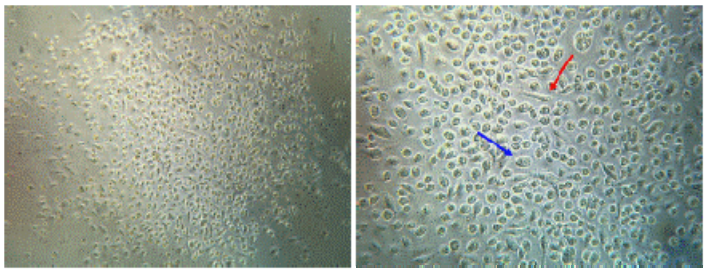

Fig1. Human blood derived-MSCs: the predominant cells consist of fibroblast-like cells.

Table1. Flow cytometry analysis: Immune phenotype of culture-expanded MSCs..

Antigen	CD number	Expression
HCAM-1	CD44	Positive
Endoglin	CD105	Positive
SB10/ALCAM	CD166	Positive
HCAM-1	CD34	Negative

DISCUSSION & CONCLUSIONS: *In vitro* induction of chondrogenesis has been demonstrated in both bone marrow and peripheral blood-derived MSCs using 3-dimensional scaffold producing comparable cellular expressions. MSCs which are easily isolated (and less painfully harvested) from peripheral blood as compared to bone marrow provides a superior alternative source for MSCs for future clinical application (in this case for cartilage repair).

REFERENCES: Heng B. C., Cao T. and Lee, E. H. (2004) Stem cells **22**, 1152–1167. Hirsch, M. S., Lunsford L. E., Trinkaus-Randall, et al (1997) Dev. Dyn. **210**, 249–63.

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The Attachment of Human Primary Osteoblast cells to Oxygen Plasma Modified PEEK

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Introduction: Polyetheretherketone (PEEK) has come into the spotlight as a replacement for metals in devices such as spine cages and craniomaxillofacial (CMF) implants, due to its high strength, good wear and radiolucent properties[1]. Evaluation of soft and hard tissue integration to implants by X-ray or MRI can be obscured by the presence of the metal devices. Several implants have therefore been redesigned into this radiolucent material. However, cellular attachment to polymers such as PEEK is restricted due to their intrinsic low surface energy, which can lead to implant loosening, as a result of fibrous encapsulation. Higher energy surfaces have been shown to promote rapid cellular adhesion and spreading, in contrast to surfaces with lower energy[2,3]. To improve cell attachment the surface energy can be increased by plasma surface treatment. The present study aims to investigate the effect of oxygen plasma treatment of PEEK on the attachment and functionality of primary human osteoblast-like cells (HOB).

Materials and Methods: Injection moulded PEEK Optima™ discs (Invibio) with a 13mm diameter were modified by radio frequency (RF) plasma treatment, Thermanox (THX) (Nunc) and Ti ISO 5832/2 (Synthes) were used as the control surfaces. Using an EMITECH RF plasma treater, the samples were exposed to varying treatment times. Surface chemical compositions of treated and untreated surfaces were characterised by XPS, wettability by contact angle; topographic changes by AFM and SEM. HOB cells isolated from femoral heads removed during total joint replacement operations were grown to 70-80% confluence in DMEM (10% FCS in 5% CO_2 at 37°C), and plated at 10000 cells/cm^2. Alpha-MEM (0.1µM dexamethasone and 10mM beta-glycerophosphate) was used as mineralisation media over 21 days. Cell functionality was assessed by alkaline phosphatase expression (ALP), mineralisation by Alizarin red S (ARS) staining of calcium, cell attachment by SEM and cell density through the alamarBlue™ assay.

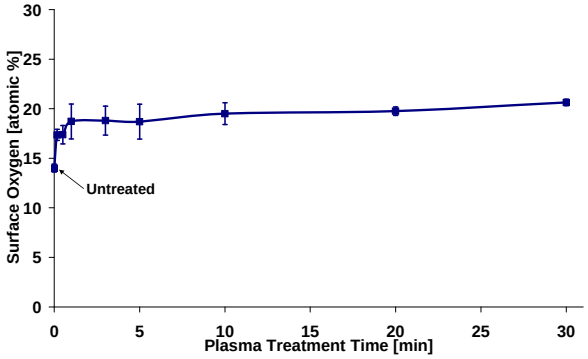

Figure 1: *Surface oxygen concentration of PEEK surfaces with increasing plasma treatment time.*

Results: XPS analysis of the untreated PEEK discs showed 14 atomic% surface oxygen, as expected, indicating that these surfaces are relatively hydrophobic in character[4]. Analysis of the plasma treated PEEK surfaces showed that the surface oxygen concentration increased with increasing treatment time up to ~20 atomic% (Fig 1). High resolution C1s spectra showed a greater increase in C-OR type functional groups than C=O and O-C=O with increasing treatment time. To study the effects of the surface treatment on cell attachment and functionality, the cells were observed after plating on the treated and untreated PEEK, THX and cpTi surfaces. Within 72hrs, the treated surfaces were shown to have higher cell densities than the untreated surfaces (Fig 2). By day 21 the treated surfaces were shown to have similar cell densities to cpTi. Oxygen plasma treatment has been found to etch polymer surfaces, by AFM, this was found to occur after more than 20min plasma treatment.

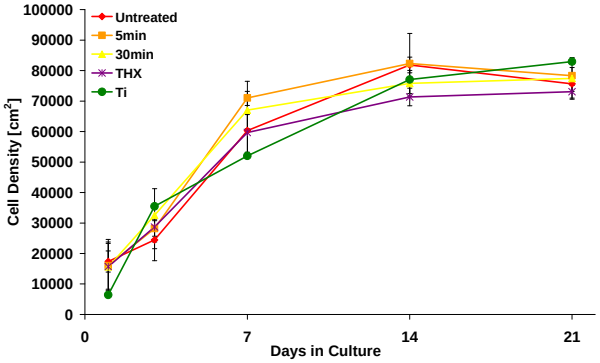

Figure 2: *HOB cell densities over the 21 days of culture, showing the optimised levels of surface treatment in comparison to THX and cpTi.*

Cells were also observed by SEM to attach more readily to the treated surfaces with higher concentrations of C-OR functional than the untreated surfaces. ALP expression was observed to be more characteristic on the PEEK surfaces with higher concentrations of C-OR functional groups. Nodule formation quantified by dissolving the ARS stain was found to be greater on the PEEK surfaces than on the THX surfaces, and similar to the levels on the cpTi surfaces.

Discussion/Conclusions: The incorporation of oxygen through plasma treatment can be used to increase the surface energy and thereby aid the adhesion of HOB cells. Surface treatment of PEEK has led to higher levels of nodule formation than on THX and similar levels to cpTi, indicating that these treated surfaces are likely to improve bony integration to implants.

References: [1]Kurtz, S.M. and Devine, J.N. Biom., 28, 4845, 2007. [2]Lopez, G.P., Ratner, B.D., et al. J Biom. Res, 26, 415, 1992. [3]Kasemo, B. Surf. Sci. 500, 656, 2002. [4]Comyn, J., Mascia, L., and Xiao, G. Int J Adhesion & Adhesives, 16, 97,1996.

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In vitro study of UHMWPE/MWCNT – Preliminary results

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INTRODUCTION: Reinforcement of ultra high molecular weight polyethylene (UHMWPE) by adding multiwalled carbon nanotubes (MWCNT) allows improvement of mechanical characteristics for biomedical applications. However, there is controversy when it comes to carbon nanotubes toxicity [1-4].

METHODS: Osteoblast-like MG63cells were seeded on 12-well plates (7600 cells/well). Polyethylene particles and polyethylene with carbon nanotube reinforcement particles were suspended in growth medium in a concentration of 500µg/mL and added in triplicate to cells. The control group was cultured in growth medium only. These were cultured for 6 days, renewing medium and suspensions every 48 hours. All experiments were run three times, at least in triplicate. After 144 hours, the culture supernatant was removed and WST-1 reagent (BioVision) in medium added and cells incubated. Supernatants were centrifuged, transferred to 96-well plate and read at 450 nm and at 655nm. Cell lysates were obtained by use of Triton 0,1% and sonication and total protein measured using the BCA method (Calbiochem). For statistical analysis, because data were not normal, the Kruskal-Wallis test was used.

RESULTS: The microscopic observation of cultured cells shown morphological changes in the cells cultured with polyethylene particles: supranuclear vacuolization, a more spindle-like shape and suggestion of engulfed particles, at times. However, at 144 hours all groups had reached confluence.

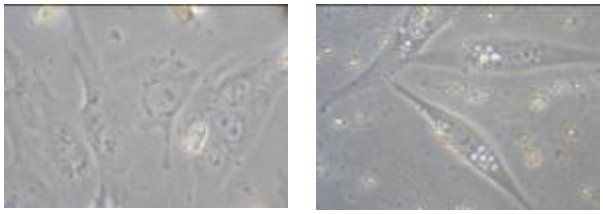

Fig. 1: Cells after 24 h in contact with composite nanoparticles (left) (400X) and polyethylene particles (right) (200X).

According to the WST-1 results there was no significant loss in viability.

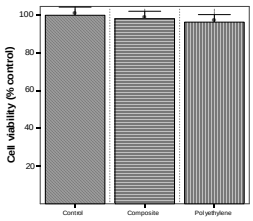

Fig. 2: Cell viability measured by the WST-1 assay (n=6). Results are expressed in percent related to untreated controls.

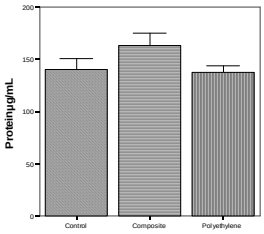

Fig. 3: Protein measured using BCA assay (n=92). Mean ± SEM.

Total protein values also failed to show significant differences between groups. However, protein measured was higher in the nanocomposite wells.

DISCUSSION & CONCLUSIONS: Although further studies are necessary and are being undertaken, the present results show good biocompatibility of the nanocomposite, comparable to results obtained by other authors. [2, 4]

REFERENCES: [1] G. Jia G., H. Wang, L. Yan et al. (2005), *Environ Sci Technolo*. **39**:1378-1383. [2] J. Chlopek, B. Czajkowska, et al.(2006), *Carbon* **44**:1106-1111. [3] J. Meng, L. Song et al. (2006) *J Biomed Mater Res* A **79**:298-306. [4] K. Pulskamp, S.Diabate et al *Tox Lett* **168**: 58-74.

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SCAFFOLD FREE GENERATION OF INTER VERTEBRAL DISC USING ROTATIONAL CULTURE SYSTEM

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INTRODUCTION

Tissue engineering methods to generate an artificial intervertebral disc has been considered a possible treatment option. Generation of a tissue using various tissue engineering methods often rely on cells and scaffolds. Recent tissue engineering studies claim that scaffold free techniques may generate better tissue formation. In articular cartilage repair, three-dimensional chondrocyte-plate, and suitable tissue-engineered cartilage was constructed without the use of scaffold. [1, 2] However, to date, there has been no report of generation of an annulus fibrosus or a nucleus pulposus tissue without the use of cell supporting scaffold. The aim of this pilot study was to investigate neither whether it was possible to form a disc tissue without the use of any scaffold nor additional growth factors using a novel technique, with a combination of static culture and rotational culture systems.

METHODS

Annulus fibrosus (AF) cells were isolated from intervertebral disc tissue of Japanese white rabbits (n=10) by enzymatic digestion and then expanded in monolayer culture. After AF cell cultures were passaged once, cells were cultured at primary static cultures for 7 days and then either cultured 2 weeks for another static culture or rotational culture. Nucleus Pulposus (NP) cells were isolated from intervertebral disc of Beagles (n= 4) by enzymatic digestion and then expanded in monolayer culture. After NP cell cultures were passaged once, cells were cultured at primary static cultures for 7 days and then cultured 1 or 2 weeks in rotational culture (Figure 1).

Primary static culture

AF and NP cells were trypsinized off the dish and applied to form a plate by using a mold with a diameter of 25 mm. Cells were suspended in DMEM/F12, FBS20% and 50 μg/ml ascorbic acid having a cell density of 2.5×10^6 cells/cm^2 and the cell suspension was inoculated in the mold. After 3 days, the cell suspension in the mold showed cell aggregation, an AF or NP cell plate. Then the mold was removed. The AF or NP cell plates were cultured under primary static culture condition for 7 days to form regular cylindrical shape.

Rotational culture group

After primary static culture, the plate was cultured under dynamic condition, rotational culture(70 rpm), for 2 or 4 weeks in AF cell plates and rotational culture(40 rpm), for 1 or 2 weeks in NP cell plates.

Static culture group

After primary static culture, the plate was cultured under static condition for 4 weeks in both AF and NP cell plates.

Constructed AF and NP plates were evaluated for histological analysis by Hematoxylin and Eosin staining and Safranin-O staining. Immunohistochemical staining was also performed for Type I and II collagen. In AF cell plates, biochemical analyses were also performed for proteoglycan (PG), collagen and DNA content.

RESULTS

After 2 or 4 weeks of rotational culture, AF cells formed a plate with good stability that allowed 3-dimentional handling. The rotational culture group was intensely stained with Safranin-O in comparison with static culture group. The rotational culture group was intensely stained with Safranin-O at 2 or 4 weeks (Figure 2a-c). The PG content of rotational culture group showed significant increase compared to the static culture group The DNA content of static culture group also showed increase compared to the static culture group. The content of collagen did not show significant difference in all groups.

After 2 weeks of rotational culture, NP cells formed a plate with enough stability that allowed 3-dimentional handling with surgical pincers (Figure 2d). The rotational culture group was intensely stained with Safranin-O in comparison with static culture group (Figure 2e and f). Result of collagen Ⅱ expression showed that both the rotational and static culture group were highly stained (Figure 2g and h).

DISCUSSION

AF and NP cells were both capable of forming a 3-dimensional IVD like tissue without the use of any scaffold. Both tissue generated with the use of rotational culture presented the most intense Safranin-O and collagen Ⅱ staining, suggesting that biomechanical influence of rotational culture system may become a useful method for IVD tissue engineering. Since the native AF and NP tissue possess complex matrix integrity, the relevance of scaffold free AF and NP tissue remain unclear. However, these results may be useful in development of a future technique to delay the irreversible progress of disc degeneration.

Figure 1. Methods of rotational culture and static culture

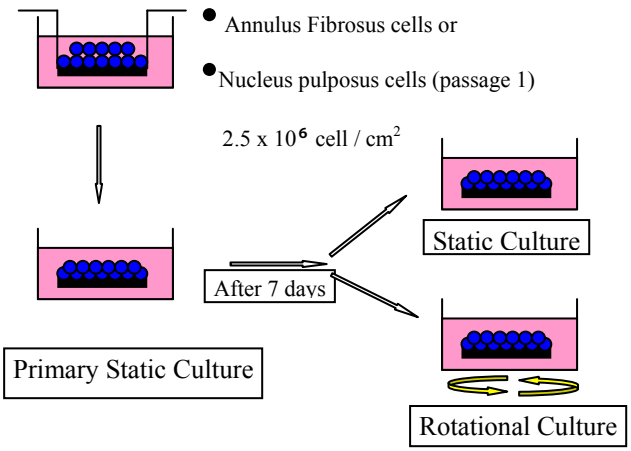
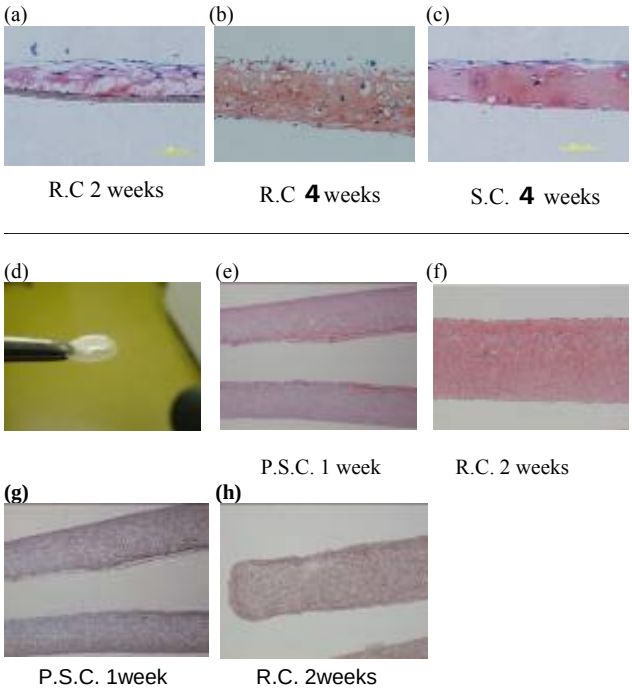


Figure 2. Safranin-O staining



REFERENCES:

[1] Nagai T et al, Tissue Engineering 2008, in press.

[2] Furukawa K et al. TESHI 2004

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Effect of TGFβ$_1$, BMP-2 and hydraulic pressure on chondrogenic differentiation of bovine bone marrow mesenchymal stromal cells ondrocytes

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INTRODUCTION: Bioactive factors such as TGFβ$_1$ and BMP-2 as well as mechanical factors i.e. compressive loading and hydraulic pressure, have been shown to induce and/or modulate chondrogenesis of bone marrow derived mesenchymal stromal cells (BMSCs). Although TGFβ$_1$ and BMP-2 belong to the same superfamily of ligands, they bind to different receptors (TGFβ receptor and BMP-2 receptor, respectively) which lead to activation of different receptor regualted-SMAD molecules More recently, Huang et al[l] have shown that cyclic compressive loading leads to up-regulation of endogenous TGFβ gene expression as well as upregulation of type I and type II TGFβ receptors. Hence, these factors are intracellularly transduced through different mechanisms and it is hypothesized that TGFβ$_1$, BMP-2 and hydraulic pressure may act synergistically on chondrogenic differentiation of BMSCs.

METHODS: BMSCs were isolated from three calves. After expansion, aggregates of BMSCs were cultured under serum free conditions in the presence of 10 ng/ml TGFβ$_1$, 50 ng/ml BMP-2 or both. Half of the samples were loaded for 4 hours per day with 0.5 to 3 MPa cyclic hydraulic pressure at 1 Hz. After 14 days of culture/loading, gene expression of collagen type I and II, aggrecan and Sox 9 was assessed. DNA as well as glycosaminoglycan (GAG) content of the pellets were analysed relative to day 1.

RESULTS Neither pressure nor BMP-2 had an influence on gene expression or GAG/DNA content. However, cells responded to the presence of TGFβ$_1$ with an up-regulation of chondrogenic genes and GAG/DNA of the aggregates increased compared to controls demonstrating the cells ability to respond to external stimuli (figure 1).

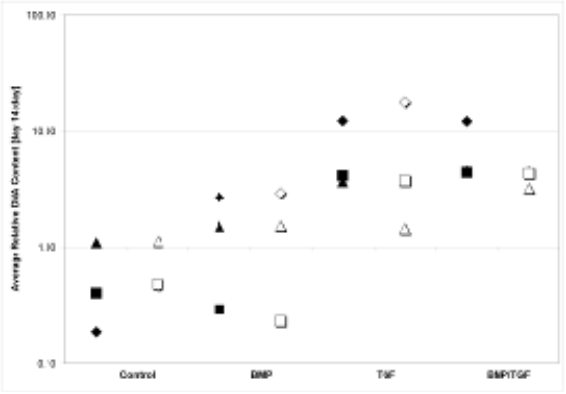

Fig. 1: GAG/DNA content of bovine BMSCs at day 14 relative to control day 1; solid symbol represent unloaded samples, unfilled symbols loaded samples; (n=3); logarithmic scale.

DISCUSSION & CONCLUSIONS: The upregulation of chondrogenic genes in the presence of TGFβ$_1$ confirms that cells within our system were able to respond to an external stimulus. BMP-2 and hydraulic pressure were neither able to induce nor modulate chondrogenesis in contrast to previous work by others. However, the effect of growth factors is dependent on many factors such concentration and duration of exposure, cell type, cell source, culture system and species of donor.

Several studies have shown only limited to no induction of chondrogenesis, if hydraulic pressure only was used. On the other hand compressive loading lead to an upregulation of chondrogenic genes in BMSCs even in the absence of TGFβ. This may indicate a higher sensitivity of BMSC to compressive loading than to hydraulic pressure. The involvement of the TGFβ pathway in mechanotransduction of compressive loading has been demonstrated[l]. To our knowledge the mechanotransduction of hydrostatic pressure in BMSCs has not been investigated yet. Differences in how mechanical stimulus is transduced might explain the differences between studies.

REFERENCES: [1] C.Y Huang, P.M. Reuben, and H.S. Cheung (2004) *Stem Cells* **23**(8):1113-1121.

Experimental Characterization of Pressure Wave Generation and Propagation in Biological Tissues

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INTRODUCTION: This work develops a measurement technique for pressure waves in soft tissues. It aims at characterizing, understanding and modeling the generation and propagation of pressure waves in soft tissues, with a view towards medical applications for the treatment of various tissue pathologies.

METHODS AND RESULTS: First, Hopkinson bar techniques[1] have been adapted to measure the waves emitted by an impact generator, transmitted through the material and reflected at the interfaces. Simulation with an explicit FEM software reproduce the measured data well.

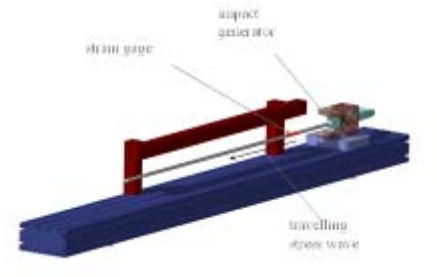

Fig. 1: Schematic of Hopkinson pressure bar and stress records at one location along the bar

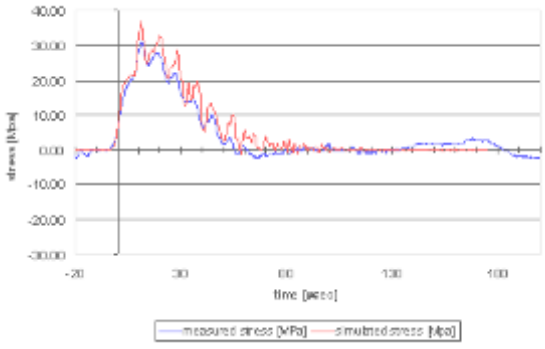

Fig. 2: measured and simulated record for an impact speed of 15m/sec

Secondly, flexible PVDF gages have been calibrated for measurement of wave propagation in soft tissues. Although PVDF gages have acoustic impedance similar to soft tissues, they act nevertheless as foreign inclusion and perturb the measurements. FEM simulation using a non linear hyper-elastic Ogden model (1) to represent soft tissues can predict the behavior of wave propagation in biological material.

$$\phi = (2\mu/\alpha^2)(\lambda_1^{\alpha}+\lambda_2^{\alpha}+\lambda_3^{\alpha}) \quad (1)$$

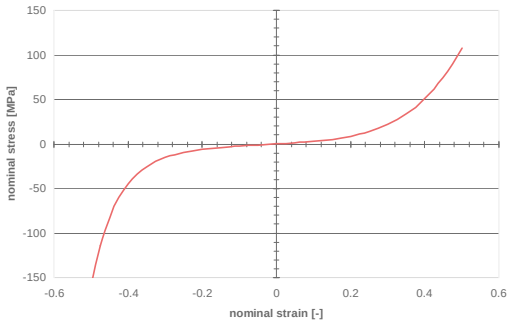

Fig. 3: Ogden model for an incompressible, isotropic, hyper-elastic solid and nominal stress-strain response

Comparison of results of simulations with and without the gage inclusion provides a method to evaluate the measured signals and extract reliable pressure data. Results are applied to the design of biomedical devices for the Extracorporeal Shock Wave Therapy (ESWT).

DISCUSSION & CONCLUSIONS: Adapted Hopkinson bar technique coupled with FEM simulation can predict pressure wave emitted, transmitted and reflected at the interfaces of an impact generator. PVDF flexible gages are a potential technique for measuring wave propagation in soft tissues and non linear hyper-elastic Ogden model is a good candidate for FEM simulation of wave propagation in these tissues. This work contributes to improve the scientific knowledge of the healing effect of shockwaves for different pathologies by providing a well characterized mechanical wave input in the treated tissues.

REFERENCES: [1] H. Kolsky (1963) *Stress waves in solids*, New York, Dover publications, Inc.