Introducing the Concept of the 3Rs into Tissue Engineering Research

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Summary

Tissue engineering, defined as using a combination of cultured cells and biodegradable scaffolds to repair tissue damaged by injury or disease, represents a booming sector of biomedical research. Animal experimentation is routinely performed prior to clinical trials. The presented study tries to translate the aspect of the 3Rs to tissue engineering research: Cell culture protocols were adapted to antibiotic free and serum free conditions. Biomaterials (Bio-Gide® and a collagen sponge prototype) were pre-tested using the HET-CAM assay. CAM-testing suggested a protocol change for application of the Bio-Gide® scaffold and demonstrated unsuitable material properties of the collagen sponge. Application of 3R compliant protocols for tissue engineering research led to increased cell proliferation, higher synthesis of extracellular matrix molecules, reduced dedifferentiation and more information about the biomaterials at an early experimental stage. Tissue engineering research can therefore profit from the increased efforts to validate in vitro alternatives and supplements to animal testing.

Zusammenfassung: Biomedizinische Forschung aus dem Blickwinkel der 3R betrachtet

Die Technik des „Tissue Engineering" repräsentiert einen boomenden Zweig der biomedizinischen Forschung. Menschliche Zellen werden isoliert, expandiert und auf eine Matrix aufgebracht, um geschädigtes Gewebe zu regenerieren. Die hierfür eingesetzten Tierversuchsprotokolle wurden am Beispiel eines Experiments zur Meniskusreparatur im Sinne der 3R überarbeitet: Antibiotika- und serumfreie Zellkulturtechniken kamen zum Einsatz, und die als Matrix dienenden Biomaterialien (Bio-Gide® und ein Kollagenschwamm-Prototyp) wurden mit Hilfe des HET-CAM Tests evaluiert. Die HET-CAM Ergebnisse führten zu einer Änderung des Protokolls für die in vivo Evaluierung der Bio-Gide® Matrix und ergaben unbefriedigende Materialeigenschaften für den Kollagenschwamm. Die 3R-konforme Überarbeitung der Versuchspläne führte zu verbesserter Zellvermehrung, gesteigerter Synthese extrazellulärer Matrixmoleküle und verringerter Dedifferenzierung der Zellen. Durch den Einsatz des HET-CAM Tests konnte zusätzliche Information über die Biokompatibilität der Implantate gewonnen werden. Dies zeigt, dass die Bestrebungen zur Validierung von Ersatz- und Ergänzungsmethoden zu Tierversuchen auch in der biomedizinische Forschung Anwendung finden.

Keywords: tissue engineering, the 3Rs, serum free cell culture, antibiotic free cell culture, HET-CAM

1 Introduction

Tissue Engineering has emerged as a new field in the biomedical sciences. Twenty-five years ago scientists believed that human tissue could only be replaced either by transplantation of other tissue from a donor or with fully artificial materials. Today, ambitions have moved from simple replacement towards reconstruction of the original tissue. Tissue engineering is defined as "the application of knowledge and expertise from a multidisciplinary field to develop and manufacture therapeutic products that utilise a combination of matrix scaffolds with viable human cell systems, or cell responsive biomolecules derived from such cells, for the repair, restoration or regeneration of cells or tissue damaged by injury or disease" (European Commission, Health & Consumer Protection Directorate, 2001).

Tissue engineered products are usually based on autologous cells – cells derived from the same individual's body – expanded *in vitro* and, once new tissue of the desired phenotype has been formed, re-implanted into the patient. Traditional animal models for tissue engineering research include rats, mice, guinea pigs, hamsters and rabbits. For most surgical applications, large animal models are used for their anatomical and physiological similarities to man. In 1986 the European Community passed a directive on the protection of animals used for experimental and other scientific purposes (Directive 86/609/EEC; Louhimies, 2002) stating that the commission and the member states should actively sup-

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port the development, validation and acceptance of methods which could reduce, refine or replace the use of laboratory animals. The concept of replacement/reduction/refinement (Russel and Burch, 1959, reprinted 1992) should lead to a replacement of experimental animals by nonsentient organisms, a reduction of the total number of experimental animals needed and refined testing protocols leading to less distressing experimental procedures.

Total replacement of animal experimentation for tissue engineering research seems impossible at the moment. Yet, there are several possibilities for reduction or at least refinement: Standard cell expansion *in vitro* is performed using culture media supplemented with foetal calf serum (FCS). Besides ethical concerns regarding the harvesting of FCS (Shah, 1999; Jochems et al., 2002; van der Valk et al., 2004), traces of serum in the transplanted cells may cause an immune reaction in the host, leading to antibody formation. The use of serum free culture conditions might pose an alternative. Also, the use of growth factors to promote cell proliferation for tissue engineering applications is controversially discussed (van Tienhoven et al., 2001). The package insert of Carticel® cultured autologous chondrocytes states that in 43% out of 81 treated patients hypertrophic tissue was noted in the follow-up arthroscopy (package insert of Carticel®). Finally, a serum free cell culture protocol not requiring the supplementation of growth factors might be an advantage. The routine antibiotic supplementation of culture media may mask latent infections (Kuhlman, 1993) and eventually lead to transplantation of an unsterile product. Allergies to antibiotics are a potential risk for the recipient of tissue engineered products, and they are not restricted to man (Ndiritu and Enos, 1977; Gauchia et al., 1996). Antibiotic supplementation of cell culture media should therefore be discontinued prior to implantation of a tissue engineered product.

Animal experimentation for the evaluation of safety and efficiency of biomedical devices in Austria is regulated by the National Federal Law concerning experimentation on living animals (1989). Although most research institutions perform numerous *in vitro* tests to ensure biocompatibility prior to animal testing, the implantation of biomedical devices without obtaining preliminary data is possible under the current legislation. The HET-CAM (Hen Egg Test – Chorioallantoic Membrane) test, originally validated for toxicity and irritation studies (Kalweit et al., 1987; Spielmann, 1995), is gaining increasing attention for biomaterial evaluation (Borges et al., 2003; Jux et al., 2003). Cells and biomaterials selected for animal experimentation could be studied using the HET-CAM model first to exclude unsuitable setups and to optimise experimental procedures prior to animal testing (Falkner et al., 2004; Eder et al., 2005). The aim of the presented study was to translate 3R aspects to tissue engineering research and to test the feasibility of 3R compliant procedures in biomedical application development. The experimental setup for a meniscus regeneration experiment planned in a sheep model was evaluated according to the principles of the 3Rs.

2 Material and methods

2.1 Cell culture

Sheep primary meniscus cells were isolated from cadaver material accruing at the university's animal experimentation facility. The menisci were digested in collagenase type II (Invitrogen, Lofer, Austria), 0.15% (w/v), for 8 hours. The cell suspension was filtered through a 40 µm cell strainer (Becton Dickinson Labware, NJ, USA), washed in phosphate buffered saline (PBS; Invitrogen) and seeded into 25 cm^2 culture flasks. Culture flasks were coated with 2% bovine skin gelatine (Sigma-Aldrich, Vienna, Austria) for serum free cell cultures. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 0.05 mg/ml ascorbic acid (Sigma-Aldrich) and either 10% foetal calf serum (FCS; PAA Laboratories, Pasching, Austria) or 2% (v/v) Insulin/Transferrin/Selenium mixture (ITS; Sigma-Aldrich) for serum free cell culture. Antibiotic supplementation was performed using 100 U/ml penicillin and 100 µg/ml streptomycin. Serum free cultures were split at subconfluency at a ratio of 1:2 using 0.25% Trypsin-EDTA (Invitrogen Gibco, Carlsbad, USA) for cell detachment. Trypsin was incubated for 1 minute at room temperature and incubation was terminated by addition of pre-cooled soybean trypsin inhibitor (Sigma-Aldrich) at a ratio of 1:1, followed by centrifugation at 1000 rpm for 5 minutes.

2.2 Cell characterisation

Primary cells were seeded on sterile glass slides at a concentration of 10^5 cells per slide. Evaluation was performed on day one, three and five of either serum supplemented or serum free monolayer culture. Slides were rinsed in PBS and fixed with acetone for 10 minutes. Slides were then washed in distilled water and stained with Hemalaun/Eosin (H/E) to monitor dedifferentiation, and Azan, Alcian Blue, Safranine O and with specific antibodies against collagen type I and II for characterisation of the primary cultures. All staining protocols were performed according to Romeis (1989). Immunohistochemistry was performed using goat anti-human collagen type I and type II antibodies (Abcam, Cambridge, UK). Slides were washed in PBS, incubated in 0.3% H_2O_2 to block endogenous peroxidase reactions following incubation in 2% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) and 10% (v/v) horse serum (PAA) to block non-specific reactions. The specific antibodies were diluted 1:100 in PBS containing 2% (w/v) BSA and incubated over night. Samples were washed in PBS and incubated with biotinylated horse anti-goat IgG (Abcam; Cambridge/UK) for 30 minutes, followed by washing in PBS, incubation in ABC-Reagent (Vectastain®, Vector Inc., Peterborough, UK) and antibody detection using diaminobenzidine (Sigma-Aldrich) diluted to 0.06% (w/v) in PBS containing 0.5% Triton X-100 (Sigma-Aldrich). 10 µl 30% H_2O_2 were added per 100 µl diaminobenzidine solution.

For three-dimensional cell culture, $5x10^5$ cells of passage 2 were pelleted by centrifugation at 1200 rpm for 10 minutes. The pellets were cultured for two weeks prior to histological analysis.

2.3 Antibiotic free cell culture

Antibiotic free cell culture was performed in a standard research laboratory. Cells were maintained in a separate incubator. Laminar air flow, water bath and incubator were washed and sterilised monthly. The humidity-sensor was changed every other month. The devices were scrubbed with S&M Laboratory Granulate (Schülke & Mayr, Austria), rinsed with distilled water and dried. All parts were sprayed with Biotenside® (Arkana, Austria) and dried. The laminar air flow was soaked in Biotenside® over night and left to air dry. The water bath was treated the same way. The incubator was ventilated and sterilised using the device's self-sterilising programme. The effects of antibiotic free cell culture were monitored via growth curves and histological characterisation. To evaluate cell proliferation, cells were plated in 24 well plates (1.9 cm^2 per well) at a density of 10^4 cells per well. Assessment of cell counts was performed daily in triplicates by detaching the cells using Trypsin-EDTA 0.25% (Gibco Invitrogen), staining with Trypan Blue 0.4% (Sigma-Aldrich) and counting the viable cells in a Neubauer haemocytometer. Routine mycoplasma testing was performed using in situ DNA fluorescence with 4'6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Vienna/Austria) according to the supplier's protocol.

2.4 HET-CAM assay

Fertilised, specific pathogen free White Leghorn (*Gallus domesticus*) eggs were supplied by Baxter Vaccine AG (Orth a. d. Donau, Austria) on incubation day 4. The egg shells were disinfected and 2 ml albumen was removed as previously described (Falkner et al., 2004). The egg shell was opened and the amniotic membranes were excised to expose the developing CAM in a way accessible to treatment. The egg shell was closed with tin foil and the eggs were incubated in a standard cell culture incubator set to 37°C, 5% CO_2 and 95% humidity. A commercially available collagen type I/III scaffold for tissue regeneration (Biogide®; Geistlich, Wolhusen, Switzerland) and a collagen sponge prototype (Geistlich) intended for meniscus restoration were tested using the HET-CAM assay. The scaffolds were aseptically cut into 25 mm^2 pieces and moistened in either FCS supplemented cell culture medium or PBS prior to transplantation onto the CAM.

All HET-CAM experiments were performed in triplicate and repeated on two separate occasions. Samples were applied on incubation day 7 and maintained *in ovo* for three days. Embryo viability was controlled hourly for the first 12 hours after sample application and at 12 hour intervals afterwards. Digital documentation was performed every 24 hours. The embryos were killed after specimen explantation according to animal welfare procedures by opening all major blood vessels and freezing at -20°C (Falkner et al., 2004). The CAM area carrying the implant was completely processed for histological analysis: CAM specimens were fixed in 4% (w/v) neutral buffered paraformaldehyde at 4°C and embedded in Paraplast (Shandon, Runcorn, UK). 5 µm serial sections were prepared and stained in Hemalaun/Eosin according to Romeis (1989).

3 Results

3.1 Serum free cell culture

Sheep meniscus primary cells maintained under serum free conditions started to attach to culture flasks after 24 hours and were securely attached after 48 hours. Proliferation was slower than in FCS supplemented cultures. Population doubling times of the primary cultures were 30.5 hours for FCS supplemented and 57 hours for serum free cultures. The morphologic appearance of the cells was different under the different culture conditions: FCS supplemented meniscus cultures demonstrated three phenotypes after cell isolation: Round cells, polygonal cells and spindle shaped cells were present on day 1 after isolation. After five days *in vitro*, only the polygonal fibroblast-like phenotype could be observed. Serum free primary cultures demonstrated a round phenotype, which was still present after 5 days *in vitro* (Fig. 1). Dedifferentiation towards the fibroblast-like phenotype was observed after approximately 10 days in culture.

Histological characterisation of FCS supplemented primary cultures demonstrated negative Alcian Blue and Safranine O staining, indicating the absence of glycosaminoglycan synthesis. Azan staining for non-specific collagens was

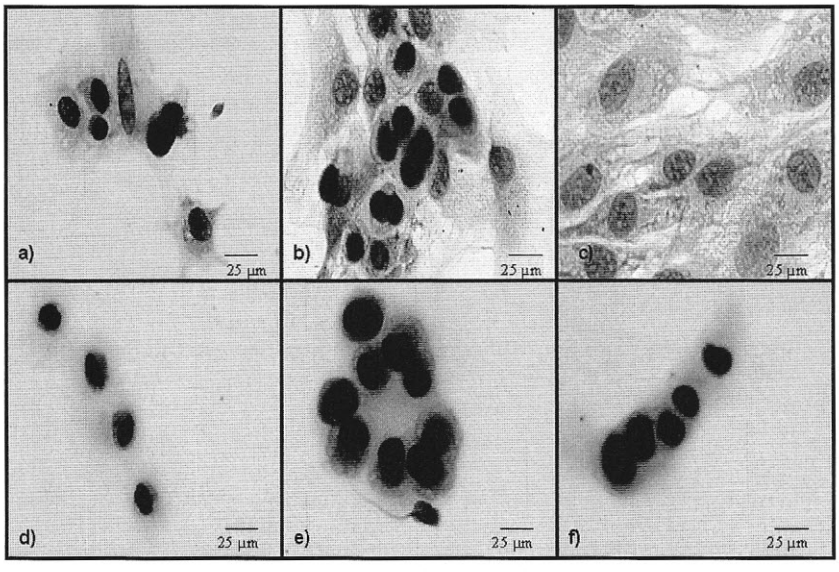

Fig. 1: Serum supplemented and serum free primary cultures (Passage 0).
a) FCS supplemented culture on the first day after isolation, demonstrating round as well as fibroblast-like cells b) same culture after 3 days c) after 5 days in culture only the fibroblast-like phenotype prevails d)-f) serum free cultures at the same time points, always showing a round, chondrocyte-like appearance. Staining: H/E (The coloured figures of this article are available on www.altex.ch).

slightly positive while immunohistochemistry demonstrated slightly positive immunostaining of collagen type I and intensive staining of collagen type II (Fig. 2 a-e). Native sheep menisci demonstrate both collagen type I and type II positive staining at comparable levels. Sheep meniscus fibrochondrocytes maintained in serum free monolayer culture demonstrated positive Alcian Blue and Safranine O staining and markedly higher levels of collagen type I synthesis (Fig. 2 f-j).

Three dimensional cell culture increased collagen type I and II synthesis in FCS supplemented cultures, so that culture results were comparable to those of serum free cultures regarding collagen type I synthesis. Collagen type II synthesis was lower than in serum free cultures and glycosaminoglycan staining remained negative (Fig. 3 a-f).

3.2 Antibiotic free cell culture

No bacterial or fungal contamination occurred during more than 12 months of culture. Routine mycoplasma testing was negative. Proliferation of serum free cultures was markedly improved by the omission of antibiotic supplementation (Fig. 4). Population doubling time was shortened to 25.6 hours. Generation times were 0.79 for FCS and antibiotic supplemented cultures, 0.42 for serum free/antibiotic supplemented cultures and 0.94 for serum free/antibiotic free cultures. The collagen content was increased and dedifferentiation – indicated by loss of collagen synthesis at higher passages – was reduced (Fig. 5).

3.3 HET-CAM assay

3.3.1 Bio-Gide® scaffold

The scaffold was securely attached to the CAM after three days *in ovo* and no obvious adverse effects such as bleeding, vessel malformation or vessel thrombosis were observed. Scaffolds moistened in culture medium containing 10% FCS prior to application onto the CAM demonstrated a significant angiogenic effect, which was not observed in scaffolds moistened in PBS only (Fig. 6 a,b). Histological analysis demonstrated good tissue integration and blood vessel formation in the surrounding of the implant (Fig. 6c).

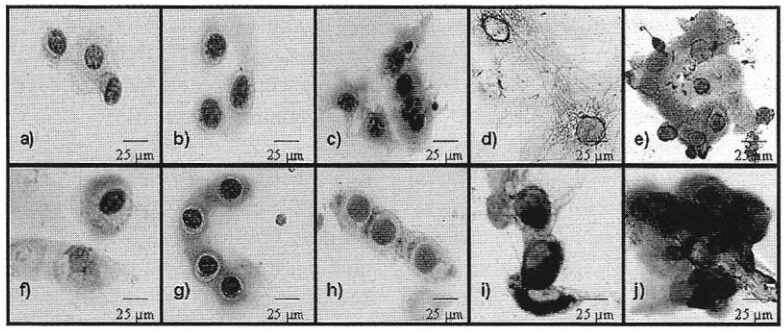

Fig. 2: Improvement of histological characteristics by serum free cell culture (Passage 0).
a)-e) FCS supplemented culture: a) negative Alcian Blue staining, b) slightly positive Azan staining, c) negative Safranine O staining, d) positive collagen type I staining, e) positive collagen type II staining. f)-j) Serum free cultured cells: f) positive Alcian Blue staining, g) positive Azan staining, h) positive Safranine O staining, i) collagen type I, j) collagen type II.

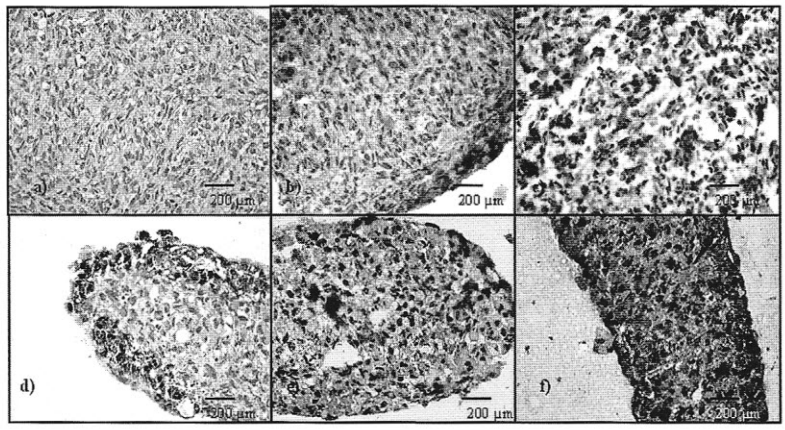

Fig. 3: Spheroid culture of sheep meniscus cells.
a)-c) FCS supplemented culture: a) negative Alcian Blue staining, b) collagen type I, c) collagen type II. d)-f) Serum free culture: d) positive Alcian Blue staining, e) collagen type I, f) collagen type II.

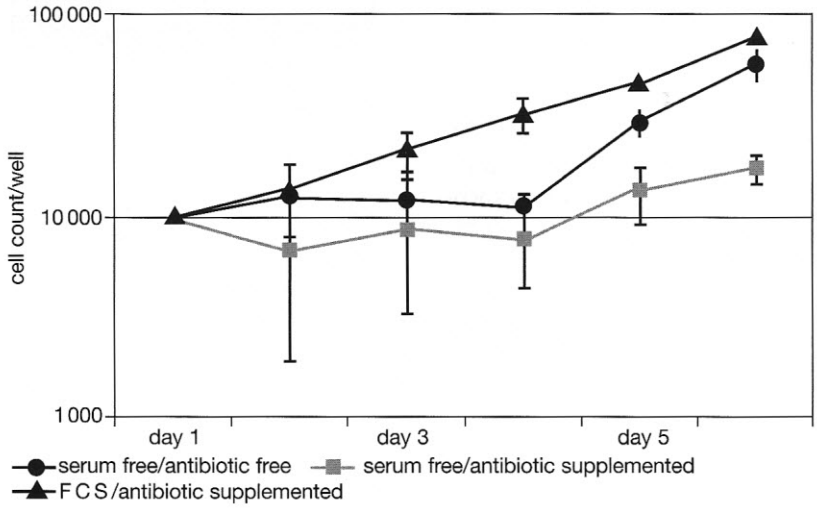

Fig. 4: Proliferation of FCS/antibiotic supplemented, serum free/antibiotic free and serum free/antibiotic supplemented primary cultures of sheep meniscus cells (Passage 0), n=3.

3.3.2 Collagen sponge

The collagen sponge prototype showed extremely rapid degradation patterns *in ovo* and could not be manipulated with tweezers for sample explantation. Spontaneous CAM bleeding and a retraction of blood vessels from the implantation site were observed (Fig. 6 d,e). Histological analysis demonstrated failed implant attachment and a foreign body tissue response (Fig. 6 f). The material was therefore considered unsuitable for meniscus tissue engineering and was not included in further experimental plans.

4 Discussion

The presented study attempted to transfer aspects of the 3Rs to tissue engineering research. Routine cell culture for tissue engineering is performed using FCS as a supplement to promote attachment and proliferation of mammalian cells. Serum is sourced as a by-product of the beef industry. The high protein diet of dairy cows may consist of meat and bone meal, which is a scientifically accepted cause of Bovine Spongiform Encephalopathy (BSE; Falkner et al., 2003). Serum used for cell cultivation *in vitro* is a major source of viral contaminants (Jennings, 1999), which often do not produce cytopathic effects or morphological changes. A great number of viruses have the potential to infect cell cultures, but only a limited number is likely to be detected in quality controlled sera (Rhangan et al., 1979; Kappeler, 1996; van der Noordaa et al., 1999).

Traces of bovine proteins deriving from FCS supplementation of cell cultures may cause allergic reactions in the recipient, as tissue engineering experiments are usually not performed using bovine animal models. FCS supplemented "autologous" cell cultures should therefore be regarded as xenografts. Looking at data obtained from human clinical trials, several studies have been published reporting allergic reactions to FCS components used for cell expansion *in vitro* (Mackensen et al., 2000; Tschuong et al., 2002; Spees et al., 2004).

The benefits of serum free culture medium are easily deducible: Serum free cell culture promotes chemically defined and controlled culture conditions (Gstraunthaler, 2003), leads towards elimination of a potential source of microbial contamination (Froud, 1999; Merten, 1999), allows the selection of specific cell types and delays dedifferentiation phenomena (Brown and Schneider, 2002; Chaipinyo, 2002; Yokoyama, 2004). Regarding serum free culture of meniscus fibrochondrocytes, only few protocols are published. Tumia and Johnstone used DMEM supplemented with 50 µg/ml ascorbic acid and various concentrations of bFGF for serum free culture of sheep meniscus cells (Tumia and Johnstone, 2004). The benefit of ITS supplementation in combination with various growth factors was demonstrated

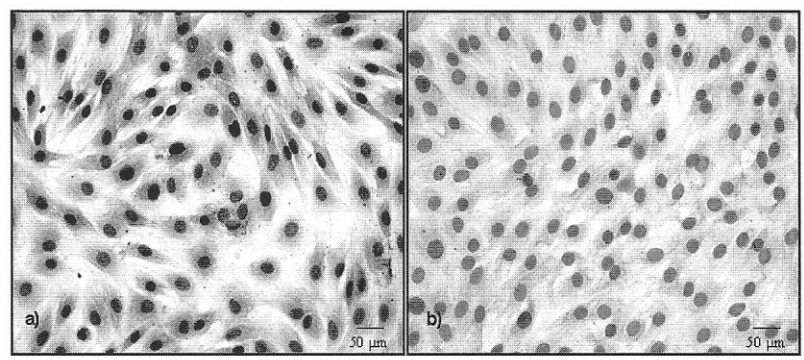

Fig. 5: Cells cultured in medium containing 10% FCS with and without antibiotic supplementation.
Passage 5 cells maintained in antibiotic free medium stain positive for collagen type I (a) while corresponding cells cultured in antibiotic supplemented medium demonstrate negative staining results (b).

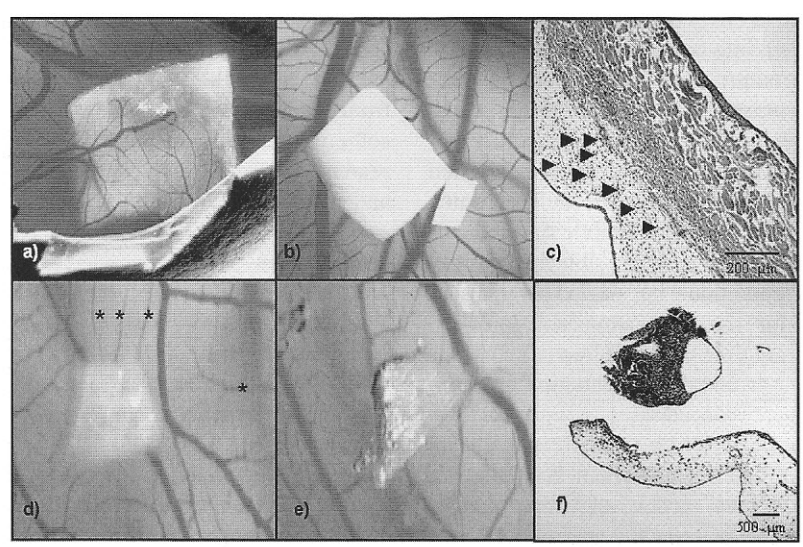

Fig. 6: HET-CAM testing results of the Bio-Gide® scaffold and the collagen sponge prototype.
a) Bio-Gide®, angiogenic response caused by traces of culture medium used for scaffold moistening after 3 days *in ovo* b) Bio-Gide®, absent angiogenic response after scaffold moistening in PBS after 3 days *in ovo* c) Bio-Gide®, histological analysis demonstrating good tissue integration and the formation of blood vessels in the surrounding of the scaffold (arrows); staining: H/E. d) Collagen sponge on day 1 after sample application; stars marking blood vessels present on day 1 but absent on day 3 after sample application; e) collagen sponge after 3 days *in ovo*: vessel bleeding and anti-angiogenic effect; f) histology demonstrates failed tissue integration and a foreign body tissue response; staining: H/E.

for chondrocytes by various authors (Chua et al., 2005; Morgan et al., 2005). The presented study tested serum free primary culture of meniscus cells without addition of specific growth factors.

Serum supplemented primary cultures isolated from meniscus fibrocartilage contained a large number of cells with a phenotype different from fibrochondrocytes. After 5 days *in vitro*, these cultures demonstrated a unique, fibroblast-like appearance. Under serum free conditions, cultures demonstrated a round phenotype for the first 10 days of culture as well as synthesis of extracellular matrix proteins typical for chondrocyte-like cells. As meniscus cells derive from a poorly vascularised tissue, serum free culture conditions might reflect a more physiological environment.

Although antibiotic supplementation is standard practice in cell culture routine and clinical applications, the uncritical use of antibiotics carries several disadvantages: Besides induction of resistant bacterial strains by insufficient dosage or incorrect waste disposal and the possibility of side effects or allergic reactions in case of cell transplantation, the antiproliferative effect on all living cells should be taken into account (Kuhlmann, 1993). As a routine research laboratory is hardly comparable to an automated GMP compliant tissue engineering facility, antibiotic free cell culture was performed under non-GMP conditions. The results demonstrated that antibiotic free cell culture was not only possible under non-GMP conditions, but carried several benefits for the cell cultures: Population doubling time of serum free cultures was decreased and dedifferentiation phenomena were reduced.

The HET-CAM test offers a cheap, simple and 3R compliant borderline *in vitro/in vivo* model, allowing the pre-evaluation of cells and scaffolds prior to actual animal experimentation (Falkner et al., 2004; Eder et al., 2005). Using the HET-CAM test for pre-evaluation of biomaterials designed for tissue engineering, the Bio-Gide® scaffold demonstrated impressive angiogenic properties when applied after pre-moistening in culture medium. Applying scaffolds soaked in PBS as control indicated that the angiogenic effect was due to the FCS in the culture medium and not to material properties. A change in protocol was therefore suggested for later animal testing of the Bio-Gide® scaffold to avoid traces of culture medium influencing experimental results. The collagen sponge prototype demonstrated rapid *in ovo* degradation, failed tissue integration and it elicited a foreign body tissue response. The biomaterial was therefore considered inappropriate and excluded from further experimental procedures. Considering the financial, personnel and time requirements of animal experimentation, biomaterial pre-testing using the HET-CAM bioassay is not only desirable in terms of the 3Rs, but also beneficial for the researcher.

The presented study tried to transfer aspects of the 3Rs to tissue engineering research. All starting points tested turned out to be not only favourable in terms of the 3Rs, leading to refinement and reduction of animal experiments, but also favourable in terms of the scientific experiment, leading to better cell proliferation, higher synthesis of extracellular matrix molecules, reduced dedifferentiation phenomena and adding additional information about biocompatibility at an early experimental stage. Tissue engineering research can therefore profit from the increased efforts to standardise cell culture technologies and *in vitro* research and to validate *in vitro* alternatives and supplements to animal testing.

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