A modified HET–CAM approach for biocompatibility testing of medical devices

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Abstract

The implantation of new biomedical devices into living animals without any previous toxicity or biocompatibility evaluation is possible under current legislation. The HET–CAM (Hen Egg Test–Chorionallantoic Membrane) test offers a partially immunodeficient, borderline in vitro/in vivo test system that allows the simulation of transplantation experiments to obtain biocompatibility data prior to animal testing. A collagen type I/III scaffold, designed for tissue regeneration, was tested for angiogenetic properties and biocompatibility patterns. A significant angiogenetic stimulus caused by the collagen scaffold material was observed. Altering biocompatibility patterns by incubation with the potentially hazardous chemicals acridine orange and ethidium bromide led to severe vessel thrombosis and a foreign body tissue response. CAM testing of biomaterials and tissue engineered products allows selection of the most suitable biomaterial and the elimination of unsuitable materials from animal experiments, leading to a refinement of testing procedures and a reduction in the number of animals required for biocompatibility testing.

Keywords: *animal welfare, biocompatibility testing, chorionallantoic membrane, HET–CAM test, Three Rs, tissue engineering*

Introduction

Tissue engineering has emerged as a new field of medical science. Twenty-five years ago scientists believed that human tissue could only be replaced by either transplantation of other tissue from a donor or with fully artificial materials. Both approaches have their associated problems: transplantation is limited by organ shortage and immunological concerns, whereas the inert implantable devices can rarely replace the structure and function of natural tissues. For a long time, the construction of a bioartificial tissue — a hybrid created from a combination of living cells and natural or artificial polymers — was thought to be impossible. Tissue-engineered products are usually based on the use of autologous cells — cells derived from the same individual's body — expanded *in vitro* and, once new tissue of the required phenotype has been formed, re-implanted into the patient. Epithelial cells, or combinations of keratinocytes and fibroblasts, have been successfully used as a source of tissue-engineered skin (Briscoe *et al* 1999), chondrocytes for cartilage reconstruction (Ochi *et al* 2001) and osteoblasts for bone repair (Davies 2000). Patient derived stem-cells obtained from bone marrow and differentiated into the appropriate phenotype *in vitro* are being increasingly studied (Fukuda 2001).

Careful biocompatibility assessment is crucial prior to Food and Drug Administration (FDA) approval and the clinical application of tissue engineered products. The authors, van Tienhoven *et al* (2001) investigated the pre-clinical safety assessment of tissue engineered medical products and listed several potential hazards related to biomaterial implantation. Variation in the source of natural 'scaffold' materials, or inconsistency in the production of raw or synthetic materials, could lead to batch variability. Systemic acute, subacute and chronic toxicity must be assessed prior to approval for clinical application. Irritation and sensitisation studies, including delayed hypersensitivity reactions, must be performed and any adverse effects related to processing residues must be assessed. New biomaterials must also be tested for pyrogenicity, blood incompatibility, genotoxicity and potential carcinogenicity.

The biological evaluation of medical devices within the European Union is regulated by the International Organisation for Standardisation (ISO) 10993 (Bohnsack 2003), which is accepted as the European standard. The use of animal experimentation, for the evaluation of safety and efficiency of new biomedical devices, is regulated by the Austrian Federal Law concerning the use of living animals in experimentation (1989) and needs authorisation by a commission. To obtain authorisation researchers must

complete an application form that requires information about the institution and staff, the number and type of animals intended to be used, the current state of research in the field of study, the scientific goals and the test procedure. The anaesthesia, operation technique, and criteria for mandatory termination of an animal experiment because of severe animal suffering, must also be described; however, no statements concerning preliminary *in vitro* testing or biocompatibility patterns of implant devices are required. The implantation of biomedical devices into living animals without any preliminary data regarding toxicity or biocompatibility is therefore possible based on the current legislation.

The HET–CAM (Hen Egg Test–Chorionallantoic Membrane) test was originally validated for toxicity and irritation studies (Kalweit *et al* 1987; Spielmann 1995) and is under increasing attention for biomaterial evaluation purposes (Borges *et al* 2003; Jux *et al* 2003; Falkner *et al* 2004). The chorionallantoic membrane (CAM) is an extra-embryonic membrane of a fertilised and incubated chicken egg; it is highly vascularised, but not innervated. Therefore, CAM testing is not considered to be animal experimentation if experimental procedures are terminated prior to incubation day 12, at which time closure of the neural duct occurs and the embryo is capable of feeling pain (Hamburger & Hamilton 1992).

The highly vascular nature of the CAM, and the rapid development of the connective tissues and the blood vessel system, offer a complex test system for tissue reaction studies. The aim of this study was to test the suitability of the CAM as a testing environment for cells and biomaterials prior to animal experimentation. A possibility for pre-evaluation of new material prototypes would allow the exclusion of inadequate materials and reduce the total number of experimental animals required.

Materials and methods

CAM preparation

Fertilised specific-pathogen-free White Leghorn (*Gallus domesticus*) eggs were supplied by Baxter Vaccine AG (Orth an der Donau, Austria) on incubation day 4. The egg shells were disinfected and 2 ml of albumen was removed using a method described by Falkner *et al* 2004. The egg shell was opened and the amnion membranes were excised to expose the developing CAM in a manner accessible to treatment. The egg shell was closed using tin foil and the eggs were incubated in a standard cell culture incubator set at 37°C, 5% CO_2 and 95% humidity.

Cell culture

Sheep meniscus fibrochondrocytes were used for the simulation of cell transplantation onto the CAM. Primary cells were isolated from cadaver material provided by the Core Unit for Biomedical Research, Medical University Vienna. The menisci were digested in 0.15% collagenase type II (Invitrogen, Lofer, Austria) for 8 h. The cell suspension was filtered through a 40 μm cell strainer (Becton Dickinson Labware, NJ, USA), washed in phosphate buffered saline (PBS; Invitrogen, Lofer, Austria) and seeded into 25 cm² culture flasks. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Lofer, Austria) supplemented with 0.05 mg ml⁻¹ ascorbic acid (Sigma-Aldrich, Vienna, Austria) and 10% foetal calf serum (FCS: PAA Laboratories, Pasching, Austria).

Scaffold preparation

Commercially available collagen type I/III scaffolds for tissue regeneration (Biogide®: Geistlich, Wolhusen, Switzerland) were cut into 25 mm² pieces and soaked in PBS prior to transplantation onto the CAM. Passage two sheep meniscus fibrochondrocytes were seeded onto the scaffolds at a concentration of 10⁶ cells per 25 mm² or suspended in 100 μl PBS at the same concentration. To evaluate the effect of potential hazardous agents, sheep mensicus cells were incubated with acridine orange and ethidium bromide (VWR International, Vienna, Austria) — both of which are suspected carcinogens — according to standard protocols for live/dead viability staining of cell cultures. A stock solution was prepared from 50 μg ethidium bromide and 15 mg acridine orange dissolved in 1 ml of ethanol (96%). Distilled water was added to make the volume up to 50 ml and the solution was diluted 1:100 prior to staining. Cells were stained for 5 min at room temperature and washed in 5 portions of PBS prior to use. Acridine orange and ethidium bromide tagged cells were tested as 1) a cell suspension and 2) seeded onto a scaffold prior to application onto the CAM.

Sample application

All experiments were performed in triplicate and repeated on two separate occasions. Samples were applied on incubation day 7 and maintained *in ovo* for 3 days. The scaffolds were carefully placed on top of the CAM. Embryo viability was controlled hourly for the first 12 h after sample application and then at 12 h intervals. The CAMs were digitally documented every 24 h. The application site of the cell suspension was marked with a sterile silicone ring to facilitate sample identification and explanation. Again, documentation was performed every 24 h. The embryos were killed after specimen explanation by opening all major blood vessels and freezing at –20°C, according to animal welfare procedures (Falkner *et al* 2004).

Sample evaluation

The area of the CAM carrying the implant was completely processed for histological analysis: the CAM specimens were fixed in 4% paraformaldehyde at 4°C and embedded in paraplast. 5 μm serial sections were prepared and stained in hemalaun-eosin, according to Romeis (1989). For scanning electron microscopic evaluation, samples were fixed in 2.5% glutaraldehyde, drained in a graded series of ethanol from 30% to 100% and chemically dried using hexamethyldisalazane. Angiogenesis was judged by quantification of blood vessel formation in the area of the CAM carrying the implant. Thirty serial sections of the CAM

Figure 1
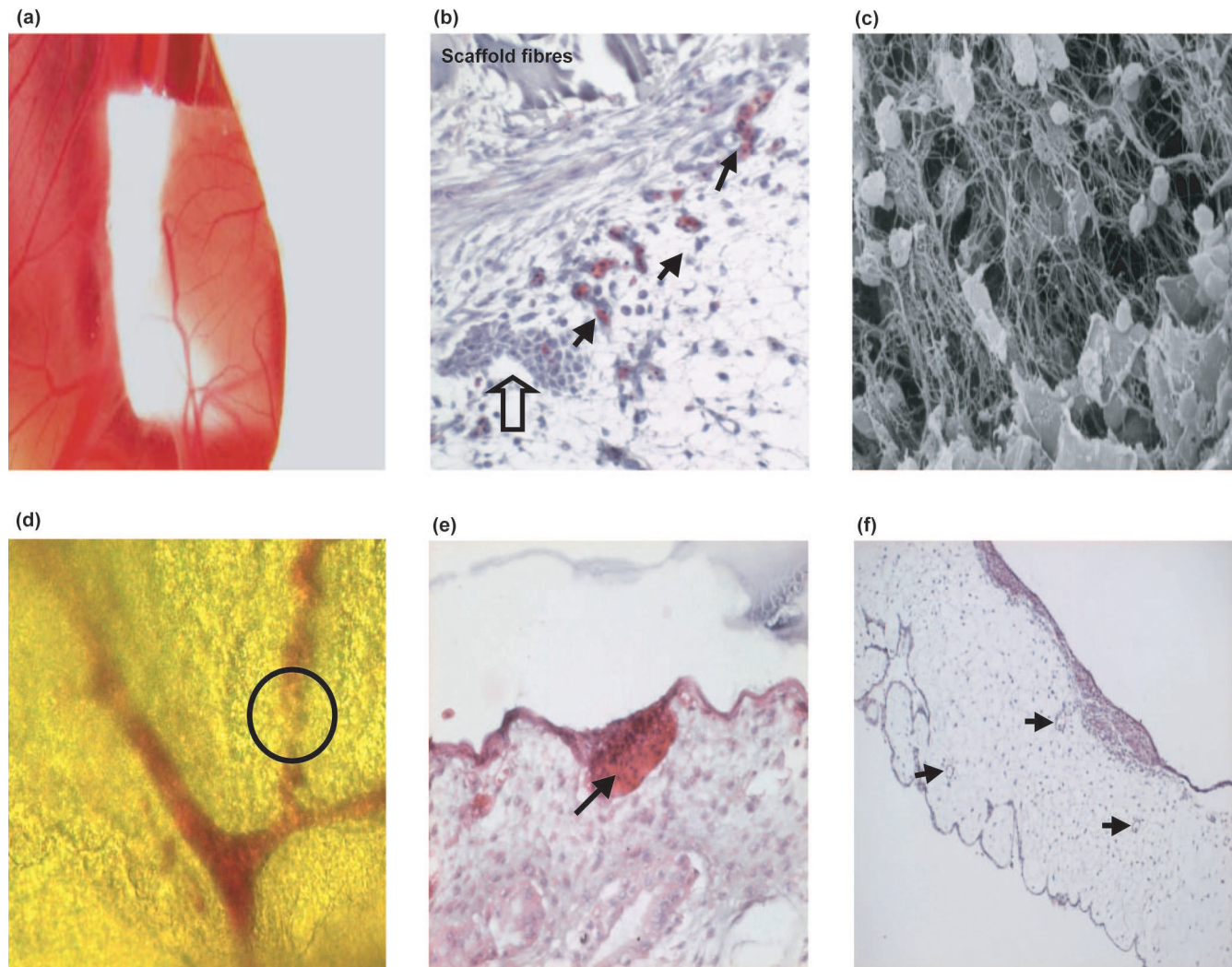
CAM testing results of cells and biomaterial. **(a)** Macroscopic aspect of the collagen type I/III scaffold after 3 days *in ovo*. Biocompatibility is indicated by the absence of a tissue response, vessel thrombosis or bleeding. Blood vessel growth into the biomaterial suggests a connection to the embryonal blood stream. **(b)** Histological section of a CAM explant. The biomaterial is firmly attached to the CAM and blood vessels can be observed sprouting in the surrounding of the implant (black arrows). The transplanted cells (white arrow) have survived and started tissue formation. Specimens stained in haematoxylin-eosin (HE), original magnification × 200. **(c)** SEM analysis shows immigration of the transplanted cells into the recipient's connective tissue, original magnification × 1500. **(d)** Macroscopic and **(e)** microscopic alterations by changed biocompatibility patterns of the biomaterial: blood vessel thrombosis (within the circle in **(d)**, and indicated by the arrow in **(e)**) and an inflammatory tissue response can be observed after incubation with acridine orange and ethidium bromide. In **(e)**, staining in HE, original magnification × 200. **(f)** Contact with acridine orange and ethidium bromide did not alter the biocompatibility of a cell suspension, as indicated by the absence of a tissue response and thrombotic vessel reactions (arrows indicating open blood vessels). Staining in HE, original magnification × 100.

were prepared: one 5 μm section was prepared and the next 95 μm were discarded before the next section was collected. The CAM sections were then evaluated: the number of blood vessels that had grown into the CAM by 1 mm were counted using an ocular gauge at a 100 × magnification; average blood vessel content per mm of CAM was also calculated.

Blood vessel thrombosis — defined as the formation of a blood clot within the circulatory system — was used as an evaluation criterion for assessing adverse effects of cell or biomaterial implantation. Blood vessels that were completely occluded by an erythrocyte thrombus were defined as being 'totally occluded', whereas vessels in which the formation of a thrombus left part of the vessel cross-section open were defined as 'partially occluded'. The total blood vessel content, in addition to the number of total and partially occluded vessels, was counted in 30 CAM sections per specimen; the percentage of vessels having total or partial occlusion was then calculated. Statistical analysis was performed by using the software package SPSS 12.0; groups were compared using a two-tailed Student's *t*-test and were considered significantly different if $P < 0.05$.

Figure 2
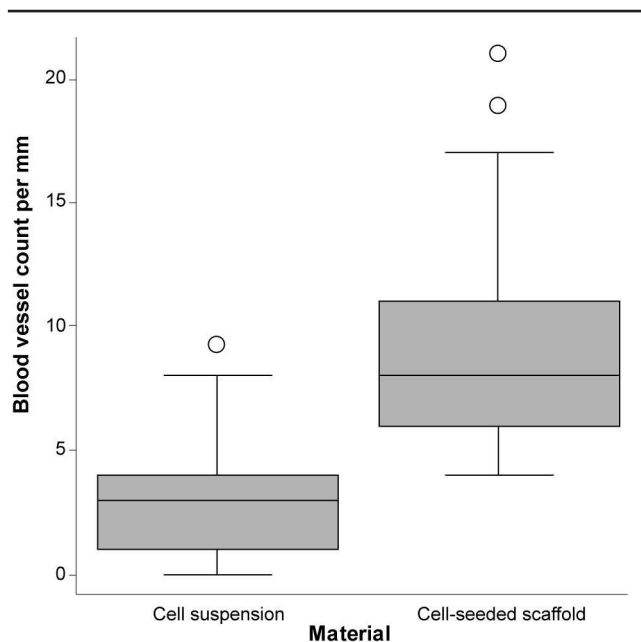
Angiogenetic CAM response to cell transplantation or the implantation of a cell-seeded scaffold. Average blood vessel count was 3.02 per mm after the application of a cell suspension and 8.90 per mm after transplantation of a cell-seeded scaffold ($P < 0.001$). The data are represented as a box plot: the box contains the middle 50% of the data, the line within the box indicating the median value. The top and bottom of each box represent the 75th and 25th percentile, respectively. The ends of the vertical lines represent the minimum and maximum data values, the open circles represent outliers.

Results

Macroscopic evaluation

Macroscopic evaluation revealed firm attachment of the implant to the connective tissue of the CAM after 3 days *in ovo* (Figure 1a). The implant did not separate from the CAM during specimen explanation, histological processing or scanning electron microscopy. Numerous blood vessels in the implant indicated a connection of the biomaterial to the embryonal blood stream. No obvious adverse effects, such as bleeding, vessel thrombosis or vessel malformations, were observed.

Microscopic and scanning electron microscopy evaluation

Histological analysis confirmed a good attachment between the biomaterial and the CAM. The scaffold was completely adhered to the connective tissue of the CAM and chick reticular fibroblasts were observed spreading between the fibrous material of the scaffold. The transplanted cells were visible as small islands in the surrounding of the implanted biomaterial. Numerous blood vessels were present in the surrounding of the implant. No adverse effects, such as vessel thrombosis, an inflammatory foreign body reaction or CAM hypertrophia, were detected (Figure 1b). Scanning electron microscope analysis showed the transplanted cells were still attached to the implant, but were starting to migrate into deeper layers of the CAM (Figure 1c).

Quantification of angiogenesis

To measure the angiogenetic properties of the scaffold biomaterial, the angiogenetic effect of cell augmented scaffolds versus cells transplanted without a scaffold were compared. Implantation of cell-seeded scaffolds led to a significant increase in angiogenesis (Figure 1b, Figure 2): the average blood vessel count was 8.90 per mm after transplantation of a cell-seeded scaffold, but only 3.02 per mm after seeding cells without a carrier material ($P < 0.001$).

Biocompatibility patterns

To evaluate the potential of the HET–CAM system for biocompatibility testing and biocompatibility of the cells, the cell scaffold constructs were altered by incubation with acridine orange and ethidium bromide. Application of the acridine orange and ethidium bromide stained cell suspension led to partial occlusion of approximately 4% of the blood vessels, whereas total occlusion was not observed and no foreign body reaction occurred (Figure 1f). However, transplanting scaffolds seeded with acridine orange and ethidium bromide treated cells led to severe blood vessel thrombosis (Figure 1d, 1e). Approximately 22% of the chorionallantoic blood vessels were partially occluded and 7% of the vessels were totally occluded (Figure 1e, Figure 3). A foreign body tissue response showing vessel thrombosis, CAM hypertrophy and the presence of inflammatory cells was also observed. Thrombotic reaction was significantly higher ($P < 0.001$) than after implantation of the vital stained cells without a scaffold (Figure 1e, Figure 3).

Discussion

The ISO 10993 regulation (Bohnsack 2003) describes a broad spectrum of *in vitro* methods for the evaluation of toxicity, carcinogenicity, and biocompatibility of new biomaterials and tissue engineered products prior to the approval of a new product for clinical trials on humans. Expanding these regulations to animal testing would be admirable in terms of the Three Rs (Russell & Burch 1959, reprinted 1992), but appears unrealistic because of the rapid development of biomedical research. Furthermore, Universities and Institutions are likely to be unwilling, and unable, to bear the costs of additional testing and the trained personnel necessary to perform *in vitro* evaluation. The selection of a single test, which could be easily performed at the animal testing facility in combination with a basic cytotoxicity evaluation of the biomaterial using the cell type routinely handled in the specific research laboratory, might pose a cheaper and more realistic alternative.

This study tested the potential of the HET–CAM test for biomaterial pre-evaluation prior to animal testing. The transplanted biomaterials showed rapid integration into the connective tissue of the CAM and secure attachment after only 3 days *in ovo*. The scaffolds were soon infiltrated with blood vessels and showed a connection to the embryonic blood stream, indicating that the simulation of transplantation experiments — where a connection to the recipient's blood circulatory system is crucial for the survival of the implant — is possible. A 10 day old chicken embryo represents a partially immunodeficient

Figure 3
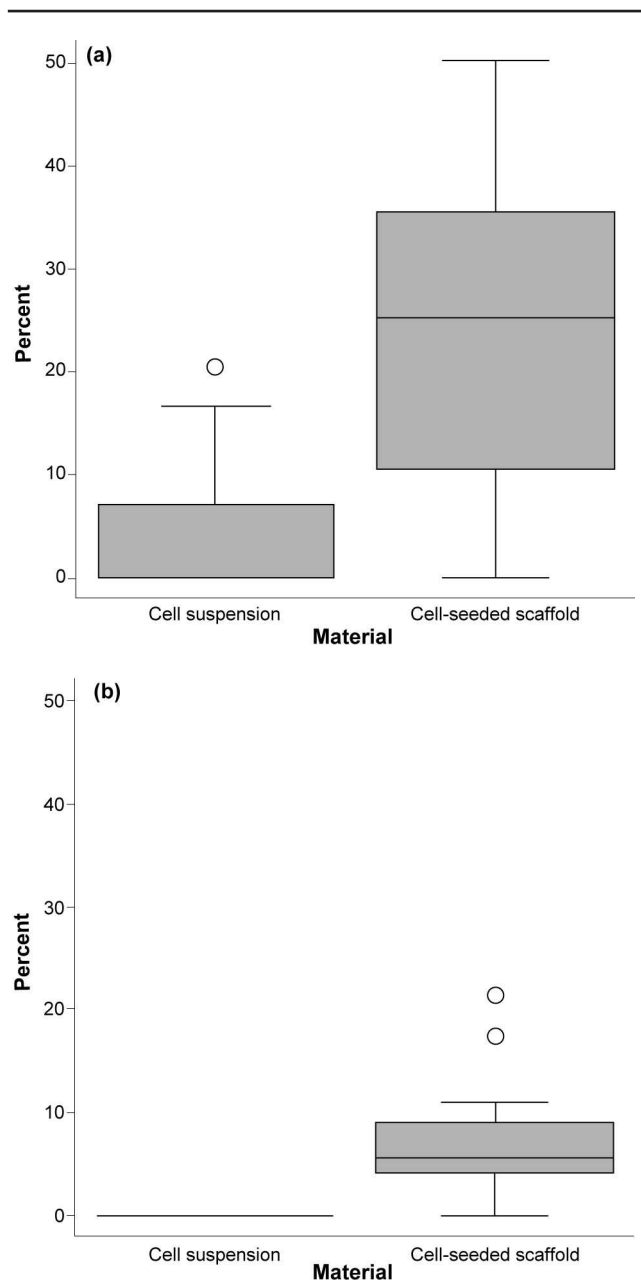
Thrombotic vessel occlusion caused by incubation of the cells in acridine orange and ethidium bromide prior to transplantation: **(a)** partially occluded and **(b)** totally occluded. Application of the cell suspension led to a partial occlusion of approximately 4% of the blood vessels; total vessel occlusion was not observed. Application of the cell-seeded scaffold led to partial occlusion of approximately 22% and total occlusion of approximately 7% of the CAM vessels ($P < 0.001$) The data are represented as a box plot: the box contains the middle 50% of the data, the line within the box indicating the median value. The top and bottom of each box represent the 75th and 25th percentile, respectively. The ends of the vertical lines represent the minimum and maximum data values, the open circles represent outliers.

model lacking a functional humoral immune response; however, a tissue reaction is possible, allowing the distinction between biocompatible and incompatible materials (Djabari *et al* 2002; Kalteis *et al* 2004).

The implanted scaffold material showed good biocompatibility patterns: good tissue integration, an angiogenetic stimulus and the absence of a foreign body reaction. The transplanted cells were able to survive *in ovo* and showed tissue formation in the surrounding of the scaffold. Incubating the cells with acridine orange and ethidium bromide — both suspected carcinogens that are used for vital staining and the discrimination of dead and living cells during *in vitro* experiments — altered the biocompatibility patterns of both the cells and the scaffold material. Severe vessel thrombosis and a foreign body tissue response were observed after the application of acridine orange tagged cell-seeded scaffolds, whereas the application of acridine orange tagged cells without a scaffold only led to mild thrombotic reaction and no foreign body tissue response.

These results indicate that CAM testing can be used to analyse the angiogenetic properties of biomaterials and provide information about biocompatibility patterns. HET–CAM is a very cheap and rapid test system (Falkner *et al* 2004) and CAM analysis can be performed in a routine cell culture laboratory; therefore, CAM testing can be used as a pre-screening of biomaterials prior to animal testing. During tissue engineering research, often more than one prototype material is developed. The routine use of the HET–CAM test would allow the exclusion of unsuitable prototypes and facilitate the selection of the most appropriate prototype for animal experimentation, therefore reducing the side-effects caused by unsuitable materials during animal experimentation and allowing the number of experimental animals required to be reduced.

Acknowledgements

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