Multiwell three-dimensional systems enable in vivo screening of immune reactions to biomaterials

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BIOCOMPATIBILITY STUDIES

Rapid Communication

Multiwell three-dimensional systems enable in vivo screening of immune reactions to biomaterials: a new strategy toward translational biomaterial research

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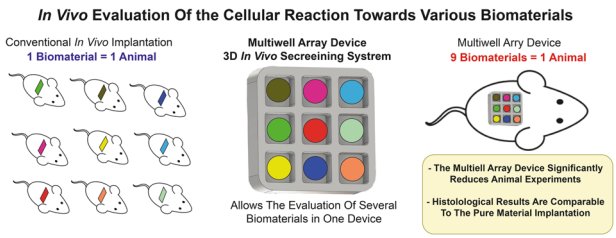
Abstract

In vivo experiments are accompanied by ethical issues, including sacrificing a large number of animals as well as large costs. A new in vivo 3D screening system was developed to reduce the number of required animals without compromising the results. The present pilot study examined a multiwell array system in combination with three different collagen-based biomaterials (A, B and C) using subcutaneous implantation for 10 days and histological and histomorphometrical evaluations. The tissue reaction towards the device itself was dominated by mononuclear cells. However, three independent biomaterial-specific tissue reactions were observed in three chambers. The results showed a mononuclear cell-based tissue reaction in one chamber (A) and foreign body reaction by multinucleated giant cells in the other two chambers (B and C). Statistical analysis showed a significantly higher number of multinucleated giant cells in cases B and C than in case A (A vs. B; $***P < 0.001$), (A vs. C; $P < 0.01$). These outcomes were comparable to previously published observations with conventional biomaterial implantation. The present data lead to the conclusion that this 3D screening system could be an alternative tool to enhance the effectiveness of in vivo experiments, thus offering a more economic strategy to screen biomaterial-related cellular reactions, while saving animals, without influencing the final outcome.

Graphical Abstract



1 Introduction

The rapid development of biomaterials introduces many different bone and soft-tissue substitutes, which increase the need for preclinical in vivo studies to achieve the translation of such materials. Previous publications have underlined the efficiency of animal experiments in subcutaneous implantation models [1–3]. In this context, various large and small animal models have been established to evaluate the tissue response to biomaterials, as well as their regenerative potential [4, 5]. Recently, our group systematically investigated tissue reaction to different collagen membranes using standardized subcutaneous implantation [6–8]. A large

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number of animals was required to understand the inflammatory pattern induced by various collagen membranes. Mainly, two different cellular reactions were observed [4]. The first type of tissue response was a mononuclear cell-based inflammatory response leading to biomaterial integration, as shown in a bilayered collagen matrix, which could be successfully translated to clinical cases [6]. The analysis of another bilayered collagen also showed a mononuclear reaction, resulting in its integration within the peri-implantation bed [6, 8, 9]. The second type of tissue response was represented by the formation of multinucleated giant cells (MNGCs) as a sign of a foreign body reaction, leading to the disintegration of the evaluated biomaterials [10–12]. This conventional in vivo concept implicates the need to sacrifice a large number of animals, which is accompanied by ethical and cost problems. These concerns also influence the in vivo experimental capacity. Although continuing efforts have attempted to optimize the in vitro models and build complex 3D systems to imitate the in vivo tissue reaction, there is still no acceptable way to replace animal models [13, 14]. Therefore, the development of in vivo techniques is desired to reduce the number of required animals and simultaneously enhance the effectiveness of the chosen animal experiments. One such innovation is the concept of a multiwell array system that allows the examination of different materials and conditions in one same animal. This system previously succeeded in analyzing pre-cultured cells under 36 different conditions by direct implantation in one animal, showing comparable outcomes to the conventional in vivo methods. The present pilot study was aimed to evaluate such an array device in combination with three collagen-based biomaterials. The principal focus was placed on the tissue reaction toward the multiwell device itself and the cellular reaction induced by the three collagen-based biomaterials within the device. Additionally, the effectiveness of this system was determined by comparing the present tissue response to previously observed results after implantation of the same biomaterials using conventional techniques [6, 10].

2 Materials and methods

The three-dimensional master device with a size of 8 × 8 × 1.9 mm comprising nine wells with dimensions of 1.1 × 1.1 × 1.6 mm was produced by means of stereolithography (EnvisionTec Perfactory, Germany). The device was designed in Rhinoceros 3D (McNeel Europe) and consists of a poly(ethylene oxide terephthalate)/poly (butylene terephthalate) (PEOT/PBT) copolymer (PolyVation BV, Groningen, The Netherlands) [13]. The Committee on the Use of Live Animals in Teaching and Research of the State of Rhineland-Palatinate, Germany, approved the implementation of this study. Subcutaneous implantation was performed according to previously described techniques [15]. In summary, the multiwell system was loaded as follows. The first row included three chambers and was filled with three different collagen-based biomaterials: collagen A, a non-cross-linked bilayered collagen matrix of porcine origin (Mucograft®, Geistlich Biomaterials, Wolhousen, Switzerland); collagen B, a non-cross-linked membrane of collagen and elastin obtained from the porcine dermis (Mucoderm®, Botiss Biomaterials, Berlin, Germany); and collagen C, a non-cross-linked, bilayered collagen-based biomaterial derived from the porcine dermis (BEGO Collagen Fleece®, BEGO Implant Systems, Bremen, Germany). The middle row had three wells that were kept empty to be used as a control. The last row was again loaded with the same three biomaterials as the first row (Fig. 1a). After intraperitoneal anesthesia of four CD-1 mice (Charles River Laboratories, Germany), the loaded device was implanted in the prepared pocket ($n = 4$ mice). Ten days after implantation, animals were sacrificed by an overdose of ketamine and xylazine, and the screening device was explanted along the peri-implant tissue and was fixed in 4% formalin for 24 h [16]. Tissue preparation for the histological analysis followed previously established standard methods [10, 17]. Briefly, the specimen were stained with hematoxylin and eosin (H&E), Azan and Movat Pentachrome. Histological evaluation was performed using an ECLIPSE 80i microscope (Nikon, Tokyo, Japan) connected to a DS-Fi1/digital camera (Nikon) on an automatic scanning table (Prior, Rockland, Mass) to present the tissue response and inflammatory pattern as previously described [18]. For histomorphometry, the total scan, which is a digitalizing method of the histological slide by automatically assembling 100–200 images of the implantation area was used. The number of multinucleated giant cells (MNGCs) and area of each membrane chamber were determined using the "annotation and measurement" options in the software NIS Elements [19]. The data are expressed as the means and average in MNGCs per mm^2. The graphical illustration and statistical analysis were performed using version 7 (GraphPad Software Inc., LaJolla, USA) and one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test ($\alpha 2 = 0.05$) according to the following p values ($^*P < 0.05$), ($^{**}P < 0.01$), ($^{***}P < 0.001$) or ($^{****}P < 0.0001$).

3 Results

Particular interest was directed to the inflammatory pattern of the multiwell device itself as well as three different collagen biomaterials with a known inflammatory reaction to compare the outcomes with already published results from

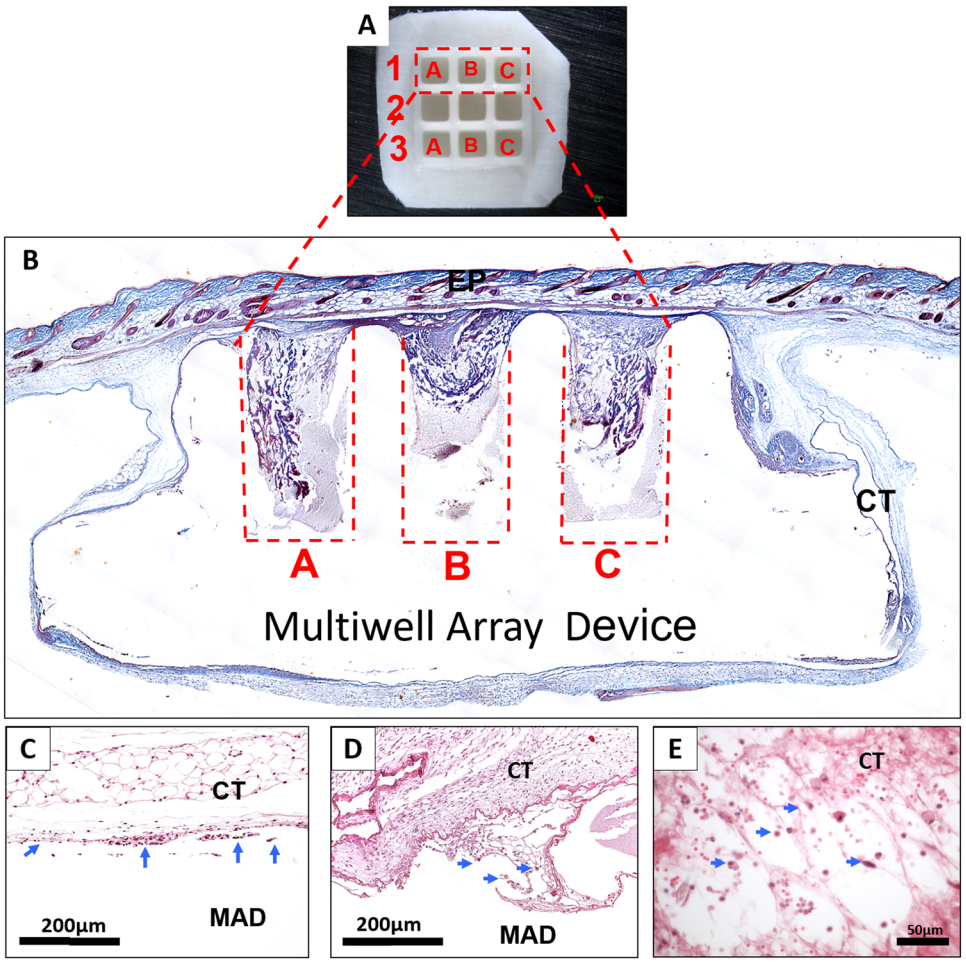

Fig. 1 **a** The multiwell 3D screening system. **b** Total scan picture demonstrates the well system on one slide including collagen (**a**–**c**). The margins of the device were used to evaluate the tissue reaction to the device itself. Host connective tissue (CT) surrounded the device and contained few mononuclear cells, Azan staining ×100 magnification. **c** The marginal area (MAD = multiwall Array Device) showing connective tissue (CT) with single mononuclear cells (blue arrows), H and E staining, ×100 magnification, Scale bar = 200 µm. **d** The tissue reaction toward the multiwell screening device (MAD) in the empty implanted chamber. Tissue ingrowth into the well system was observed. This host connective tissue (CT) exhibited few mononuclear cells (blue arrows), H and E staining, ×100 magnification, Scale bar = 200 µm. **e** High magnification of the connective tissue (CT) inside the empty chamber including only mononuclear cells (blue arrows), H and E staining, ×400 magnification, Scale bar = 50 µm

conventional in vivo methods. All animals survived implantation, and no signs of necrosis or hemorrhage were observed within the implantation site. The screening device allowed the observation of three specific tissue reactions according to the different biomaterials (Fig. 1a, b). The margin of the device, which was used as an additional control for the tissue response to the device itself, revealed no evidence of a foreign body reaction. The device was surrounded by host connective tissue containing mononuclear cells (Fig. 1c). Host tissue appeared to grow slowly into the empty chambers (Fig. 1d). The first chamber with collagen A exhibited a tissue response typified by mononuclear cells only. These cells were adherent to the surface of the collagen matrix, and single mononuclear cells were found between the collagen fibers of the membrane (Fig. 2a1–a3). The second chamber with collagen B showed a different tissue reaction. In this case, mononuclear cells were detectable on the upper part of the biomaterial and multinucleated giant cells, as a sign of a foreign body reaction, were visible on the surface (3.3 ± 0.65 MNGCs/mm^2) (Fig. 2b1–b3). In the third chamber, where collagen C was located, a larger number of mononuclear cells was observed, most of them in the form of macrophages. Additionally, multinucleated giant cells were identified on the surface of the membrane (2.8 ± 0.54 MNGCs/mm^2) (Fig. 2c1–c3). Statistical analysis showed that the number of MNGCs found within the implantation region of collagen B, as well as the number of MNGCs within collagen C, was significantly higher than that within collagen A (A vs. B; $^{***}P < 0.001$), (A vs. C; $P < 0.01$). However, no statistically significant difference was detected comparing collagen B and collagen C (Fig. 3).

4 Discussion

Animal experiments are widely used to examine different drugs, enhance the development of biomaterials and help validate the safety of novel therapies [20]. However, in vivo experiments are also associated with ethical issues and large costs. Accordingly, this pilot study introduced a new in vivo 3D screening system that enables the implantation of numerous materials into one animal. In this regard, the

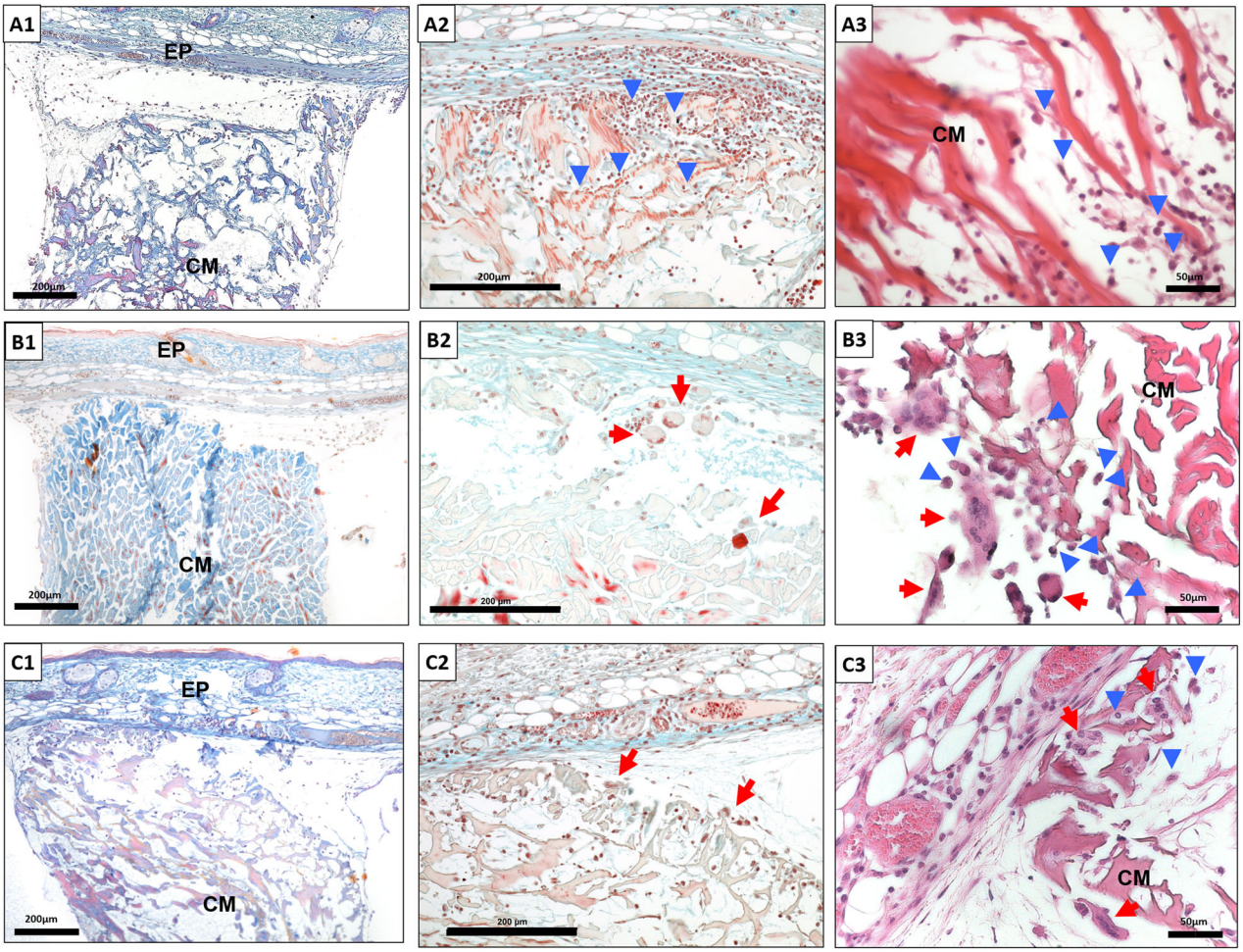

Fig. 2 **a1–3** The reaction in the first well. **a1** The host forms cell-rich connective tissue on the surface of the collagen membrane (CM) and includes mononuclear cells (blue arrow heads), (Azan staining, ×10 magnification, 200-µm scale bar). **a2** Mononuclear cells in Movat Pentachrome staining (×200 magnification, 200-µm scale bar). **a3** the central region of collagen A was penetrated by mononuclear cells only (blue arrow heads). **b1–3** The second chamber. **b1** overview of the implantation region of chamber b showing the collagen membrane B (CM) under the epithelium (EP), Azan staining, ×10 magnification, 200-µm scale bar). **b2** single multinucleated giant cells (red arrows) are detectable on the surface of the membrane in chamber B, Movat Pentachrome staining (×200 magnification, 200-µm scale bar). **b3** Multinucleated giant cells (red arrows) are clearly visible in the second well in H&E staining (×400 magnification, 50-µm scale bar). **c1–3** The third chamber. **c1** overview of the implantation region of chamber c showing the collagen membrane C (CM) under the epithelium (EP), Azan staining, ×10magnification, 200-µm scale bar). **b2** single multinucleated giant cells (red arrows) are detectable on the surface of the membrane in chamber B, Movat Pentachrome staining (×200 magnification, 200-µm scale bar). **c3** Multinucleated giant cells (red arrows) are clearly visible in the second well in H&E staining (×400 magnification, 50-µm scale bar)

device allows the implantation of up to 9 different materials or conditions in one small animals. Furthermore, ongoing development of the device may allow the evaluation of up to 100 different materials in one large animal. The present pilot study evaluated the application of only one device (3 × 3 wells). It is conceivable, that it is possible to implant more than one device in different localization in for example in rats. However, more investigations are needed to validate these approaches. Although the device accommodates different materials within a minimal space, differentiation between the tissue reaction based on mononuclear cells and a foreign body reaction by multinucleated giant cells within the adjacent chambers was possible. Thus, the observed reactions were localized in a particular interaction region with the specific collagenous biomaterials within their implantation zone. The cellular reactions to the biomaterials within the screening device correspond to the previously seen tissue responses using conventional subcutaneous implantation. Accordingly, collagen (A) i.e., the non-cross-linked collagen matrix Mucograft® has been previously investigated in the conventional subcutaneous animal model [6]. The results also indicated a reaction based on only mononuclear cells, similar to the reaction observed in the present study. Moreover, presence of multinucleated giant cells in the case of collagen (B) i.e., the thick collagen matrix Mucoderm® and collagen (C) i.e., the

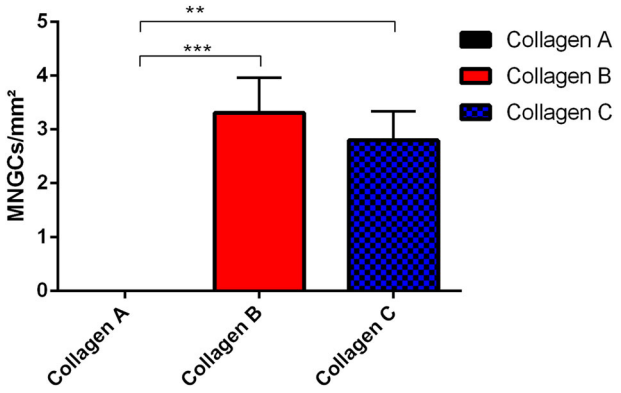

Fig. 3 Statistical analysis of the multinucleated giant cell number within the different chambers (**$P < 0.01$; ***$P < 0.001$)

BEGO collagen Fleece was previously observed in a conventional subcutaneous implantation model at the corresponding time points [7, 10]. These outcomes highlight that the screening device did not influence the tissue reaction with the collagen-based biomaterials, underlining the adequate bioinert character of this system. In this context, it is hardly possible to perform similar surgical implantation by preparing multiple pockets without connection in the same small animal. However, within the limitation of the present pilot study this approach was not tested. These findings lead to the conclusion that the implantation of more than one biomaterial in one animal allows for observation of different material-specific tissue reactions within a compromised space in the same animal. In the present study each well of the device served as a separated pocket. No interaction or crosstalk was observed. However, when implanting different biomaterials within the same animal without separation, it is very likely assumable that a crosstalk would take place and the biomaterial-induced cellular reaction may not be evaluated accurately. Using this type of system offers new strategies for biomaterial research and tissue engineering [13, 14]. The benefits are not only the implantation of diverse materials into one animal but also the possibility of a direct histological comparison of the results on one slide, as is shown (Fig. 2b). Another feature of this device is its combined in vitro and in vivo application, indicating that this system is valuable for use for cell culture and further in vivo implantation of pre-cultured materials [13, 14]. Additionally, the multiwell 3D system could serve as a tool to assess proof of concept. When comparing the number of animals used with this multiwell device to the number of animals that would otherwise be used for conventional methods, it is obvious that the application of the multiwell screening system significantly reduces the number of required animals without affecting the results (Table 1). This represents a large saving in the number of required animals. These approaches represent a responsible means of dealing with animal experiments according to the principles of the 3Rs (**R**eplacement, **R**eduction and **R**efinement) to enhance the effectiveness of animal experiments and also combines a more economical and ethically acceptable path for future research [21–23].

Table 1 A comparative overview of the number of used animals with and without the multiwell array device (MAD) to outline the significant saving of animals

Species	Mouse
Biomaterials	3
Screvo 3D system	3 × 3
Wells per animal	9
Animals with Screvo	4
Animals with conventional methods	36
Saved animals	32

5 Conclusion

The present pilot study evaluated a novel in vivo 3D screening system, which allows the implantation of numerous materials into one animal. The application of the multiwell screening device enabled the observation of two biomaterial-specific cellular reactions (mononuclear triggered vs multinucleated giant cells triggered reaction) within the same animal without interference. The outcomes were identical to the previously observed cellular reaction by conventional implantation. This 3D screening system leads to a significant reduction of the required number of animals and promotes the effectiveness of in vivo experiments, offering new possibilities for research in biomaterials and tissue engineering.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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