

Consensus Report

Fetal Bovine Serum (FBS): Past – Present – Future

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Summary

The supplementation of culture medium with fetal bovine serum (FBS, also referred to as ‘fetal calf serum’) is still common practice in cell culture applications. Due to a number of disadvantages in terms of quality and reproducibility of *in vitro* data, animal welfare concerns, and in light of recent cases of fraudulent marketing, the search for alternatives and the development of serum-free medium formulations gained global attention. Here, we report on the 3rd Workshop on FBS, Serum Alternatives and Serum-free Media, where (a) regulatory aspects, (b) the serum dilemma, (c) alternatives to FBS, (d) case-studies of serum-free *in vitro* applications, and (e) the establishment of serum-free databases, were discussed.

The whole process of obtaining blood from a living calf fetus to using the FBS produced from it for scientific purposes is *de facto* not yet legally regulated, despite the existing EU-Directive 2010/63/EU on the use of animals for scientific purposes. Together with above mentioned challenges, several strategies have been developed to reduce or replace FBS in cell culture media in terms of the 3Rs (Refinement, Reduction, Replacement). Most recently, releasates of activated human donor thrombocytes (human platelet lysates) have been shown to be one of the most promising serum alternatives when chemically defined media are not yet an option. Additionally, new developments in cell-based assay techniques, advanced organ-on-chip and microphysiological systems are covered in this report. Chemically-defined serum-free media are shown to be the ultimate goal for the majority of culture systems, and examples are discussed.

Keywords: serum-free, cell culture, databases, 3Rs, replacement

1 Introduction

1.1 Workshops on the replacement of FBS

This is the report of the 3rd workshop devoted to the replacement of fetal bovine serum (FBS) in cell and tissue culture methods. The 1st workshop, held in Utrecht, The Netherlands, in 2003 (van der Valk et al., 2004) was initiated after publication of a comprehensive report on pain and distress experienced by fetal calves during blood harvesting of their blood for production of FBS (Jochems et al., 2002). The main goal was to create awareness, to propose procedures to avoid pain and distress and to discuss possibilities to reduce or replace the use of FBS in cell culture media. A second workshop was

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organized in Copenhagen, Denmark, in 2009, to discuss current *in vitro* methods devoid of FBS or other animal components and strategies to develop serum-free media (van der Valk et al., 2010).

Since then, several new developments took place. In 2010, the revised EU Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes (European Commission, 2010) was issued, also covering fetal forms of mammals. Furthermore, in recent years there have been increased efforts towards the establishment of human platelet lysates as alternatives to FBS for use as a cell culture supplement (Bieback, 2013; Burnouf et al., 2016; Gstraunthaler et al., 2015; Hemeda et al., 2014; Shih and Burnouf, 2015). In spring 2013, a severe case of fraudulent blending of FBS batches came to public attention (Gstraunthaler et al., 2013, 2014). Also, successful serum-free applications that had been developed for primary neuronal cell systems to approximate cerebral spinal fluid (CSF) (Schaffner et al., 1995) were extended to other cell types for microphysiological systems and organ-on-chip technologies (Marx et al., 2016; Sung et al., 2013), one of which is completely serum-free (Oleaga et al., 2016). These promising developments gave reason to organize a 3rd workshop on *FBS, Serum Alternatives and Serum-free Media*.

The following current topics were discussed with experts in the field at this workshop: regulatory aspects (Section 2), the serum dilemma (Section 3), alternatives to FBS (Section 4), case-studies of serum-free *in vitro* applications (Section 5) and serum-free databases (Section 6).

1.2 Problems of FBS use

Fetal bovine serum (FBS) was introduced to stimulate cellular growth in cell and tissue culture by Theodore Puck in the late 1950s (Puck et al., 1958). It later turned out that FBS contains essential components for cell proliferation and maintenance such as hormones, vitamins, transport proteins, trace elements, spreading and growth factors (Maurer, 1986; Brunner et al., 2010). Since Puck's discovery, FBS has been used as a universal supplement in culture media for human and animal (including insect) cells in research, biotechnology and pharmaceutical manufacturing. Although bovine sera of alternative origin are available, like newborn calf serum, calf serum, and donor bovine serum, fetal bovine (fetal calf) serum (FBS/FCS) is most widely used due to its low content of immunoglobulins and complement factors (Gstraunthaler and Lindl, 2013).

FBS is collected from the blood of unborn calves (fetuses) at any developmental stage of the last two-thirds of gestation, which are mostly accidentally discovered when slaughtering pregnant cows (Jochems et al., 2002). It is calculated, that approximately 8% of cows in the slaughter line are pregnant at different stages of gestation. Recent figures estimate that about 800,000 litres of FBS are produced annually worldwide, corresponding to 2,000,000 bovine foetuses, with numbers still increasing (Brindley et al., 2012).

The use of FBS has a number of associated problematic issues. In addition to scientific disadvantages such as unknown exact composition, seasonal and geographical lot-to-lot variability (Baker, 2016b), and unintended interaction with test substances (Groothuis et al., 2015; Kramer et al., 2009), which can lead to unexpected or undesired outcomes, serious safety concerns for laboratory personnel in terms of endotoxins, mycoplasma and viral contaminants or prion proteins (Dormont, 1999; Wessman and Levings, 1999; Hawkes, 2015) as well as ethical concerns about fetal distress (van der Valk et al., 2004) and unpredictable shortages in global supply (Brunner et al., 2010; Gstraunthaler, 2003) have been raised with regard to the use of FBS.

Some of the scientific problems found to be caused by the use of FBS include the following: It has been shown that FBS can suppress transforming growth factor (TGF)- β 1-induced chondrogenesis in fibroblast-like type-B synoviocytes, inhibiting glycosaminoglycan and type II collagen production (Bilgen et al., 2007). FBS was also found to induce a more differentiated and less stable transcriptional profile in human bone marrow mesenchymal stem cells (hMSCs) compared to autologous (human) serum, as shown by genome-wide microarray analysis, which indicated upregulation of genes involved in cell cycle inhibition particularly in late passaged hMSCs cultured with FBS (Shahdadfar et al., 2005). Furthermore, high molecular weight proteins, such as albumin and globulins contained in serum, are a non-physiological environment, as somatic cells normally reside in interstitial spaces.

Batch-to-batch variability related to variations in the concentrations of serum components and their biological activity can ultimately lead to experimental variability and limit inter-laboratory reproducibility, particularly for the culture, expansion and differentiation of primary cells, representing a major cost associated with cell culture (Usta et al., 2014). Owing to the questionable reproducibility of models that rely on FBS, their use for regulatory purposes is debated (Brindley et al., 2012; Mannello and Tonti, 2007; Tekkatte et al., 2011). For this reason, serum-supplemented media should be avoided, wherever possible, and chemically defined media preferred (see also chapter 2, and van der Valk et al., 2010).

To maximize the collection of blood for FBS production per calf fetus, the blood is collected by syringe from the beating heart. According to the precautionary principle, one has to assume that there is the potential for conscious suffering during a large period of fetal development, which means that the fetal calves may consciously experience pain and discomfort during collection for FBS (Mellor et al., 2005; van der Valk et al., 2004), including being extricated from their mother's corpse, puncture of their heart and the blood withdrawal itself, which leads to their death (Jochems et al., 2002). Although recommendations have been made to avoid the suffering of the calves, there is only limited indication that these are being applied (van der Valk et al., 2004).

The employment of FBS-free media reduces animal suffering and may lead to more reproducible *in vitro* methods, some of which are developed to replace or reduce animal experiments. It therefore also contributes to the replacement, reduction and refinement of animal experiments (3Rs) (Balls et al., 1995).

Thus, safety, scientific, and ethical aspects are incentives to develop and use (fetal bovine) serum-free media.

2 Regulatory aspects

In recent years, several pieces of European legislation have addressed and encouraged the use of alternative (non-animal) methods, such as Regulation No 1907/2006 on the Registration, Evaluation, Authorisation and Restriction of Chemicals

(REACH) (EC, 2006), Regulation No 1223/2009 on cosmetic products (EC, 2009), and EU Directive No 2010/63/EU on the Protection of Animals used for Scientific Purposes (EU, 2010).

In particular, the Directive states that the use of animals in toxicology testing and biomedical research should be ended as soon as scientifically feasible to do so. While the Directive does not directly cover the issues underlying fetal serum withdrawal, the following is specified under Reason 9 of the Preamble: *“this Directive should also cover fetal forms of mammals, as there is scientific evidence showing that such forms in the last third of the period of their development are at an increased risk of experiencing pain, suffering and distress, which may also negatively affect their subsequent development. Scientific evidence also shows that procedures carried out on embryonic and fetal forms at an earlier stage of development could result in pain, suffering, distress or lasting harm, should the developmental forms be allowed to live beyond the first two thirds of their development”*.

Sera are sourced from a wide geographic range of countries, including members of the European Union (Biowest, 2016), where this Directive applies. Considering Article 1 and 3¹, it can be concluded that the harvesting of FBS from live bovine fetuses in the last third of their development, if it takes place on EU territory, is thus a procedure under the Directive because the serum is clearly used for scientific purposes and is extracted from a *living* animal. Therefore, the procedures would need to undergo a project review and authorization by a competent authority. Were the calves killed before the extraction of the blood, this would be a different case. The legal dilemma concerning this is the fact that a proper cost-benefit analysis, as required for a procedure under the Directive, would be difficult. The cost, i.e. the suffering and death of the animals involved, is completely separated from the potential, but largely questionable, benefit which might result from the continued use of FBS. Such a cost-benefit analysis would only be possible in a scenario in which the harvested FBS from specific animals would be exclusively supplied to one specific research institute for a distinct research project. Therefore, the particular benefit of the bleeding of one individual fetus remains unknown so that at present any cost-benefit considerations would not be demonstrated.

Moreover, in 2008, the ECVAM Scientific Advisory Committee (ESAC) advocated for the use of non-animal-derived supplements for *in vitro* studies wherever possible, and stated that *“for methods forwarded to ECVAM for validation/pre-validation where [the use of non-animal alternatives to serum] is not fulfilled a justification for future use must be provided, including measures taken to seek non-animal alternatives to [FBS]”* (ESAC, 2008). The drawbacks of using serum and an advice to replace it with chemically-defined (serum-free) media are also mentioned in the Good Cell Culture Principles issued by EURL ECVAM in 2005 (Coecke et al., 2005), and more recently in the workshop report on Good Cell Culture Practice (GCCP) for stem cells and stem-cell-derived models (Pamies et al., 2017). Also, the test guidelines no. 431, 439 and 492 (OECD, 2015a; OECD, 2015b; OECD, 2016) for the testing of chemicals published by the Organisation for Economic Co-operation and Development (OECD) include chemically defined/serum-free cell culture media in several *in vitro* test methods for skin corrosion, skin irritation and eye irritation testing, as listed in Table 1.

Tab. 1: Test methods described in OECD Test Guidelines (TG) and the corresponding cell culture media

Test methods in OECD TG No. 431	Cell culture medium
EpiDerm™ (EPI-200-SCT)	chemically defined medium (Liebsch et al., 2000)
SkinEthic™ RHE	chemically defined medium (De Brugerolle, 2007)
epiCS® (previously named EST-1000)	serum-free and chemically defined medium (CellSystems, 2012; Sarmiento, 2015)
EpiSkin™ SM	10 % FBS (Flamand et al., 2006; Rougier and Schaefer, 1998)
Test methods in OECD TG No. 439	Cell culture medium
EpiDerm™ (EPI-200-SIT)	chemically defined medium (Liebsch et al., 2000)
SkinEthic™ RHE	chemically defined medium (De Brugerolle, 2007)
LabCyte EPIMODEL24 SIT	5 % FBS (Kato et al., 2009, 2010)
EpiSkin™ SM	10 % FBS (Flamand et al., 2006; Rougier and Schaefer, 1998)
Test methods in OECD TG No. 492	Cell culture medium
EpiOcular™ EIT	serum-free medium (Sheasgreen et al., 1996)

¹ Excerpt of Directive 2010/63/EU (EU, 2010).

Article 1

Subject matter and scope

2. This Directive shall apply where animals are used or intended to be used in procedures, or bred specifically so that their organs or tissues may be used for scientific purposes.
3. This Directive shall apply to the following animals:
 - (ii) fetal forms of mammals as from the last third of their normal development;”

Article 3

Definitions

1. ‘procedure’ means any use, invasive or non-invasive, of an animal or experimental or other scientific purposes, with known or unknown outcome, or educational purposes, which may cause the animal a level of pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a *needle in accordance with good veterinary practice*.[...] but excludes the killing of animals solely for the use of their organs or tissue;”

The US Food and Drug Administration (FDA) has also indicated that the use of animals should be minimized and replacements utilized, including use of equivalent *in vitro* methods, when applicable (Brindley et al., 2012; Mannello and Tonti, 2007; Tekkatté et al., 2011). And, last but not least, for clinical-scale or Good Manufacturing Practice (GMP)-compliant manufacturing of cell-based products (advanced therapy medicinal products (ATMPs)) regulatory authorities request the replacement of FBS (EMA, 2011).

3 The serum dilemma

3.1 The serum market

FBS is a by-product of the beef industry and its production is dependent on a number of external factors, like weather conditions (e.g. climate changes with drought or flood) or changes in beef consumption (e.g. switch to poultry), that are hard to control (Brindley et al., 2012). The increasing use of FBS in cell culture, research, diagnostics and pharmaceutical manufacturing has led to a global business traded on the stock market. The opacity, loose regulation and near cartel-like situation of the FBS market opens doors for wrongdoing, misconduct and even fraud (Gstraunthaler et al., 2014; Hodgson, 1991, 1993, 1995).

Exact volumes of global production of FBS and availability, respectively, as well as of the worldwide demand are still unknown, which may create opportunities for abuse, mislabelling and adulteration of FBS. In 1994, it was reported that approximately 30,000 liters of “New Zealand” serum were sold worldwide. However, only 15,000 liters of high-quality FBS were collected annually in New Zealand (Hodgson, 1995). In spring 2013, another case of fraud occurred, where FBS from a global supplier appeared to be blended with bovine serum albumin, water and growth promoting additives. A U.S. Food and Drug Administration (FDA) investigation showed that 143 batches of FBS (from the years 2008 to 2013), with a total volume of approximately 280,000 liters, were affected. This incident might have had a substantial impact on the results of thousands of cell culture experiments. Also, quality and yield of cell culture-based biotechnology products, diagnostics and vaccines may have been affected (Gstraunthaler et al., 2014). As a very visible result of this turmoil in the serum market, the prices for FBS skyrocketed in 2015 from about € 80 per 500 ml up to € 1,200 per 500 ml.

Due to these latest incidents, the geographical origin, sourcing, and traceability of FBS became an issue (Siegel and Foster, 2013; Versteegen, 2016; ISIA, 2016). A crucial element of FBS quality and purity assurance lies in the traceability of the raw product. Purity-checks and back tracing of batches of FBS to the collection sites and countries of origin, respectively, can now easily be accomplished by stable isotope analysis (Ehleringer et al., 2008; Versteegen, 2016).

Therefore, in addition to ethical and scientific aspects, risk of adulterated composition and unstable prices give further reason to move away from the use of serum in general, and FBS in particular.

3.2 Reliability of scientific data using *in vitro* models, role of FBS

The use of FBS in routine cell culture is an embedded practice which is taught in almost every cell culture training. In the spirit of “never change a winning team” there appears, from a technical point of view, no need to change this methodology. Cell culture methods are intended to be highly standardized procedures to investigate changes in viability, growth and cell communication by varying single parameters such as adding chemicals of interest. However, FBS is a variable and undefined medium component with a complex composition that is still not fully understood. It may contain generally unpredictable factors, which might change between batches, for maintenance of the cell culture and impact upon responses, .

One example for such problems caused by FBS comes from translational research on cancer, where tumor cell lines are often used to model carcinogenesis. An indispensable condition for fast implementation of new clinical approaches is that researchers worldwide use identical models to generate results that can validate each other’s findings. Global Biological Resource Centres (BRCs) are equipped with authentication standards to ensure an optimal use of these models (Almeida et al., 2016; Barallon et al., 2010; Capes-Davis and Neve, 2016; Freedman et al., 2015). A recent survey revealed a reproducibility crisis in biomedical science, since more than 70% of researchers have failed to reproduce published experiments (Baker, 2016a), which in cancer research can be partly attributed to the use of different batches of FBS, as will be discussed below. Furthermore, the fast growing knowledge from intra-tumour across inter-patient to intra-cell line heterogeneities demands a comprehensive maintenance and safeguarding of a sufficient number of models to represent the genomic diversity observed across human cancers.

The qualitative and quantitative variations of FBS batches can cause selection and subclonal outgrowth of tumour cell lines. Heterogeneity of primary tumour cells represents one of the major disadvantages compared to continuous cell lines, since it is proposed that cell lines originate from a single neoplastic cell. Recently, the presence of two clones in one cell line has been described, indicating clonal evolution within a cell culture population (Quentmeier et al., 2013).

BRCs generally use high quality FBS, sufficient for proliferation of the majority of the continuous cell lines. FBS lot change exchanges at cell banks require extensive testing on a panel of cell lines representing different tissues and applications for a time period of at least 2 weeks, since one may not see the differentiating or apoptotic effects of the new FBS lot immediately after usage. Intra-cellular stores could initiate proliferation and falsify the results in short term testing, and nutritional deficiencies could cause cell lines to lose or gain genetic and phenotypic functions. The worst-case scenario is that bottlenecking selection procedures for the effects of newly purchased FBS batches on the different cell types are visible or measurable only in the rarest cases. Elimination of ancestral clones by sequential selection, possibly by unsuitable FBS charges on sophisticated cell culture, could result in replacement of novel clones which are genetically still identical, but equipped with modified features (Kasai et al., 2016). A recent comparison of experimental methods for reproducible pharmacogenomic profiling of cancer cell line panels highlighted evidence that even the amount of FBS in growth media could have an impact (Haverty et al., 2016).

An additional potential issue is associated with the use of charcoal to strip out endogenous hormones, growth factors and cytokines from serum. Charcoal stripped FBS is commonly used to study the effects of steroid hormones *in vitro*, but is prone to a high lot-to-lot variability (Sikora et al., 2016). A 2005 study found that charcoal stripping of FBS unexpectedly affected the commitment of osteoprogenitor KS483 cells, highly stimulating adipogenesis compared to normal FBS containing medium, which drives KS483 cells to differentiate into only osteoblasts (Dang and Lowik, 2005).

As a conclusion, there is an unmistakable demand for chemically defined media for achieving data reliability and for overcoming the reproducibility crisis caused by FBS charges of non-definable qualities (Baker, 2016b; van der Valk et al., 2004; van der Valk et al., 2010).

4 Alternatives to FBS

In light of the previously mentioned scientific, consistency, ethical, and supply challenges, the search for alternatives to FBS, and serum derived from animals in general, has become a major goal in the field of cell and tissue culture research (Brunner et al., 2010; van der Valk et al., 2010).

Several approaches, like serum-free media, protein-free media, animal-derived component free media (xeno-free media) and chemically-defined media are being distinguished and applied and were discussed in detail in the previous workshop (van der Valk et al., 2010).

The first widespread use of serum-free medium was developed for primary neuronal cultures to more closely approximate cerebral spinal fluid (CSF), which normally contains less than 0.001% protein compared to approximately 8% in serum (Busher, 1990). This was extended to create the first defined system where all the medium components, including the culture vessel surface modifications, in addition to the medium were well-defined (Schaffner et al., 1995). This technology has now been extended to include cardiac myocytes (Das et al., 2004; Natarajan et al., 2006, 2011), hippocampal neurons (Varghese et al., 2009), motoneurons (MNs) (Das et al., 2003, 2007a; Guo et al., 2010), sensory neurons (Guo et al., 2013; Rumsey et al., 2010), muscle (Das et al., 2006, 2007b, 2009; Varghese et al., 2009), neuromuscular junction formation (Chen et al., 2011; Guo et al., 2010; Smith et al., 2013), and a multi-organ system with functional readout (Oleaga et al., 2016).

There are now also some examples that demonstrate that adaptation to FBS-free cell culture does not have a major impact on the robustness of cell culture systems or on the scientific outcome of the studies (Jukić et al., 2016; Oleaga et al., 2016; Rumsey et al., 2013; Stancescu et al., 2015).

However, chemically-defined serum-free media have not yet been developed for every cell line or primary culture at this time (Bjare, 1992; Even et al., 2006; Gstraunthaler, 2003; Brunner et al., 2010). Additional complicating factors, such as the surface on which the cells are grown and cell preparation, might also have an effect on cell phenotypes and reproducibility.

Because of these facts, a universal chemically-defined serum-free culture medium is unfortunately not (yet) available and appears unrealistic given the high specific demand of different cell types as described below. However, with an understanding of the interplay of the factors, models with a serum-free medium have been developed for several cell types (Esch et al., 2016).

4.1 Human platelet lysates (hPL)

4.1.1 hPL in animal and human cell cultures

The long and winding road in the search for alternatives to FBS (van der Valk et al., 2004, 2010) may come to an end with the development of lysates of blood platelets or thrombocytes. Early reports in the 1980s asked why plasma and serum have different abilities to promote *in vitro* growth of cultured cells (Gospodarowicz and Ill, 1980), and found that mitogenic factors are found in serum but not in plasma (Balk et al., 1981). During the clotting process, thrombocytes are activated to release an array of α -granule factors which initiate and maintain blood clotting and promote wound healing (Blair and Flaumenhaft, 2009; Burnouf et al., 2013). Most of these factors also promote cell attachment, growth and proliferation *in vitro* (Barnes et al., 1987) (Fig. 1).

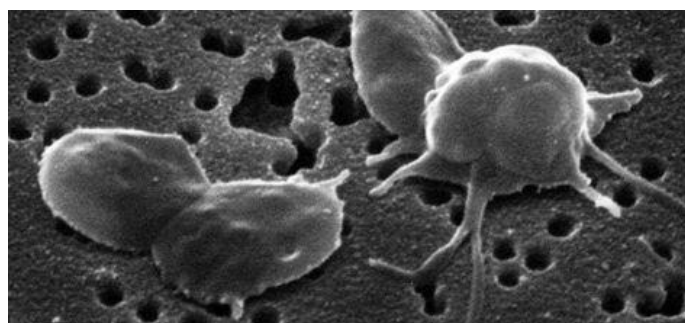


Fig. 1: Scanning electron micrograph of resting (left) and activated (right) human thrombocytes, immobilized on sterile filters

When platelets are activated, they undergo characteristic morphological changes and form long pseudopodia to adhere to the sites of vessel wall injury. In addition, thrombocytic granule factors are released by exocytosis: vasoactive peptides, factors for initiating and maintaining the clotting cascade, and growth factors for subsequent wound healing. Filters from Millipore Inc., pore size 0.22 μm . Micrograph taken by G. Gstraunthaler.

Already in 1980, human platelet lysate (hPL) or other platelet-derived factors had been introduced as promising sources of growth promoting factors for cultured cells, established cell lines, tumor cells or articular chondrocytes (Choi et al., 1980; Eastment and Sirbasku, 1980; Hara et al., 1980). Also, platelet lysate of bovine whole blood was successfully used as a general replacement for FBS in the culture of animal cell lines (Johansson et al., 2003).

hPL's are obtained by freeze-thaw of human platelet concentrates, platelet releasates (hPR) are the growth factors released after activating platelets from platelet concentrates (Bieback et al., 2009). Platelet concentrates typically are products manufactured for transfusion purposes. Accordingly, it is a well-recognized, safe and clinically tested, high quality product from certified blood donation centres. These can either be pools of buffy coat-derived platelet concentrates (typically 4 donors pooled) or apheresis-derived platelet concentrates (Fekete et al., 2012). As the original intention of platelet concentrates is for the transfusion of patients with platelet deficiency, an ethical issue may arise, when these platelet concentrates are used (instead) for cell culture. Thus it is convenient that expired platelet concentrates, after the shelf-life of maximum 5 days can be used as a cell culture supplement. Since platelet concentrates for transfusion need to be stored at room temperature, there is a risk of bacterial contamination (Pietersz et al., 2014). Thus the shelf-life (in Germany) is currently restricted to 4 days and can be extended to 5 days in case of negative bacterial testing. Typically platelet concentrates then are discarded. Now they can be saved for manufacturing cell culture supplements. Still, the risk of bacterial contamination needs to be considered. Current strategies to manage this risk are quarantine storage, although this mainly controls for the risk of donor infection. Concentrates are stored until the donors have donated blood for a second time and have been tested negative for a second time for infectious markers. The second approach, which is currently being investigated, is the use of pathogen reduction/inactivation (Castiglia et al., 2014; Jonsdottir-Buch et al., 2015; Shih et al., 2011; Stähle et al., 2010). hPL and hPR differ with respect to the growth factor content: whereas the freeze-thaw lysis to generate hPL releases all the factors contained in the platelets, including the cargo of lysosomes, dense granules and alpha granules, hPR only contains factors released in response to thrombocyte activation (the classical clotting process), thus mainly a cargo of α -granules. Depending on the activating stimulus, the composition of growth factors may differ. Interestingly, it was found that there were differences in the proliferative responses of human bone marrow derived MSCs and human adipose tissue-derived MSCs on hPL or hPR (Bieback et al., 2009, 2010; Kinzebach et al., 2013; Kocaoemer et al., 2007; Schallmoser et al., 2007; Tchong et al., 2015).

In contrast to the freeze/thawed lysate, releasates, derived from physiological activation of platelets, have undergone a clotting process, like serum in contrast to plasma. This omits the need to add anticoagulants to the cell culture. The high calcium concentrations typically present in culture media overcome the anticoagulatory effects of citrate; hPL thus requires, for instance, addition of heparin to prevent gelling (for platelet gels - see below).

The mitogenic potential of thrombocytic growth factors was determined by specific stimulation of the ERK1/2 MAP kinase signalling cascade in quiescent renal epithelial LLC-PK₁ cultures (Rauch et al., 2011). Furthermore, continuous renal epithelial cell lines were used to assess the expression of differentiation endpoints. Monolayers of cultured renal epithelia grow as highly differentiated, polarized cells (Gstraunthaler, 1988). When cultured on permeable growth supports, cells form a transporting epithelium, separating an apical and a basolateral fluid compartment. In order to generate transepithelial transport *in vitro*, some requirements must be met by the culture system: retention of the polar architecture and junctional assembly of the epithelial cells, presence of vectorial transport, sidedness of cellular uptake routes, and expression of kidney-specific metabolic and transport properties. As an overall parameter for the integrity of the cultured epithelium, the generation of transepithelial electrical resistance (TEER) can be determined (Srinivasan et al., 2015). The effect of hPL on differentiated functions of cultured renal epithelia was therefore determined. TEER was monitored in filter-grown LLC-PK₁ and in low- and high-resistance MDCK epithelia under FBS- or hPL-supplemented growth conditions. Low-resistance epithelia generated a TEER of 150-200 $\Omega \cdot \text{cm}^2$ in PL-supplemented media, as seen with FBS, and high-resistance MDCK I retained their TEER of 8,000 $\Omega \cdot \text{cm}^2$ under hPL (Gstraunthaler et al., 2012), showing that FBS can be replaced by hPL.

Lucarelli et al. (2003) and Doucet et al. (2005) were the first to suggest using platelet-derived growth factors in the culture of human mesenchymal stromal cells. With human platelet lysates (hPL), bulk thrombocytic growth factors are added to basal culture media, providing a human-based, xeno-free culture system (Gstraunthaler et al., 2015; Rauch et al., 2011). However, hPL are superior over other human-based alternatives for several reasons. Firstly, the source of origin for hPL, human donor platelet concentrates, are easily available due to their short shelf life (see below). Secondly, hPL are low protein extracts of thrombocytic growth factors, in contrast to other human serum-derived alternatives (Bieback, 2013; Burnouf et al., 2016; Gstraunthaler et al., 2015; Hemeda et al., 2014; Shih and Burnouf, 2015).

Mojica-Henshaw et al. compared hPL-serum converted from hPL plasma by recalcification with similar effects on growth of human MSC (Mojica-Henshaw et al., 2013). As different responses of MSCs and (adipose-derived stem cells (ASCs) to hPL and hPR were observed, the proteomic profiles of both were compared, which was related at least in part with the biological function of the MSCs and ASCs (Kinzebach et al., 2013). Reduced fibrinogen concentrations in hPR were also detected compared to hPL. This might be of therapeutic interest, as a recent study (Copland et al., 2013) observed that platelet lysate fibrinogen negatively affects the immunomodulatory capacity of MSC (up-regulation of indoleamine dioxygenase (IDO) and suppression of T cell proliferation).

Platelet (lysate) gels, the semisolid clot formed during platelet activation and fibrin formation, may become increasingly popular as scaffolds for reconstructive purposes, but also as 3D scaffolds for cell culture approaches (Fortunato et al., 2016; Walenda et al., 2012).

4.1.2 hPL in tissue storage and primary cell expansion for transplantation

A cognate variant of expansion of stem cells is primary cell expansion as a part of patient treatment. Both stem cell cultures and primary cell expansion share the need for cell extraction from patient tissue. Both kinds of cells have to be cultured and expanded under very specialized conditions and one of the most important aspects is constancy in their cellular characteristics

(Burnouf et al., 2016; Lee and Kay, 2006). After the *in vitro* treatment, both kinds of cells are meant to be re-transplanted into the patient to palliate symptoms or to cure the illness (Centeno et al., 2010; Parekh et al., 2016). The way from the donor/patient tissue to *in vitro* cell culture and back to the patient has to be understood as a closed circle of patient related treatment. Having this in mind the storage of human tissue is still an underestimated part of the clinical work regarding animal-free approaches to achieve benefits for the patient and the animals.

In corneal graft storage, for example, a highly regulated regime was installed to supervise and evaluate standards for the more than 40,000 operations alone in the United States and Europe every year. The North American Eye Bank Association recommends hypothermic storage and most eye banks use Optisol-GS. This medium is actually serum-free thus in cold storage metabolic activity is very low. Nevertheless, the mean storage time of 2 days is very short and leads to a high rejection rate of transplantation material. European eye banks use organ culture as storage method for corneal grafts which exceed the mean storage time up to 24 days. The organ culture media consists of fetal calf serum in a range of 2% up to 8% (Doughman et al., 2012). This media composition is still based on media used for over 50 years in cell culture. A few approaches report use of alternative media in organ or tissue storage, but none of them were brought to a new standard recommendation (Rieck et al., 2003; Smith and Johnson, 2012; Yokoo et al., 2008). In fact, all chemically defined non-serum alternatives till today do not show a better endothelial cell survival under long term storage conditions. Another obstacle for the use of serum- or animal-free media is the strict regulation of eye banks around the globe. An eye bank or a clinic switching their evaluated standard procedures risks their status as a supplier or transplantation center. Therefore, the interest in clinical studies investigating serum-free long-term storage conditions for transplantation grade corneal grafts is very low considering the fact that the established norms work and that all alternative approaches just match the old FBS standard without improvement. Furthermore, the use of transplantation material for the evaluation of alternative media faces ethical concerns.

First approaches with corneal cell lines showed a higher vitality of cells grown with a processed variant of hPL (Thieme et al., 2016). Therefore, it is now unequivocally important to test hPL in corneal graft storage, since without clinical studies showing the superiority over the old FBS media there will be no update of the existing eye bank norms regarding animal-free storage media.

Only new organ bank recommendations demanding the use of serum-free media as first choice will lead to a stepwise replacement of animal serum in organ banking. The *in vitro* culture of primary cells or stem cells with animal free media would follow naturally from such cell banking conditions.

4.1.3 hPL opportunities

To summarize, hPL appears to be a good and economical answer to the reproducibility and ethical issues related to the use of FBS. For the first time, the source of origin, animal whole blood from slaughterhouses or expired human donor thrombocytes from blood banks, is easily accessible. Both the availability and the ease of lysate preparation give hope that platelet lysate may replace FBS as universal growth supplement for highly specialized cell culture applications in the future, reviewed in: (Bieback, 2013; Burnouf et al., 2016; Gstraunthaler et al., 2015; Hemeda et al., 2014; Shih and Burnouf, 2015). As for FBS (see Chpt. 3), no figures are available for annual amounts of expired donor platelets and manufacturing capacities. As mentioned above, human donor platelet units have a shelf life of 4 days. There are estimates that 50 - 60 % of donor platelet units in stock expire and have to be discarded, which could potentially be used for hPL production (Astori et al., 2016).

Efforts for standardisation of hPL have recently been initiated. Since the number of reports on successful applications of hPL is steadily increasing over time, paralleled by an increase in the description of hPL preparation methods, there is clearly a need to reach international consensus on production, quality, and safety criteria of hPL (Astori et al., 2016; Burnouf et al., 2016). So far, the promotion of cell proliferation, the differentiation behaviour of stem cells and the cellular metabolic activity are commonly agreed parameters to prove the superiority of hPL over FBS, which are documented in many published studies.

With increasing acceptance of hPL as a valuable alternative to FBS, in recent years a market for hPL has evolved and hPL products have become commercially available. To date, 11 companies were identified, which manufacture or distribute hPL preparations (BOX 1).

Box 1: Commercial hPL products (companies)

- CLS Cell Lines Service ([http://clsgmbh.de/p16287_human_Platelet_Lysate_\(hPL\).html](http://clsgmbh.de/p16287_human_Platelet_Lysate_(hPL).html))
- COMPASS Biomedical (<http://www.compassbiomed.com/research-grade-plustrade-cell-culture-supplement-human-platelet-lysate.html>)
- COOK Regentec (<http://stemulate.cookregentec.com/>)
- HELIOS BioScience (<http://www.atcbiomed.com/UltraGRO.html>), distributed by PELOBiotech (http://www.pelobiotech.com/files/factsheet_ultragro.pdf)
- macopharma (<http://platelet-lysate.macopharma.com/>)
- Mill Creek Life Sciences (<http://www.millcreekls.com/pltmax>), distributed by MERCK MILLIPORE
- PL BioScience (<http://www.pl-bioscience.com/products/plsolution/>)
- STEMCELL Technologies (<https://www.stemcell.com/products/product-types/cell-culture-media-and-supplements/human-platelet-lysate.html>)
- TRINOVA Biochem (<https://www.trinova.de/cruxrufa.php>)
- zenbio (<http://www.zen-bio.com/products/serum/fetal-bovine-serum.php>)
- Zentrum für Klinische Transfusionsmedizin Tübingen (<http://www.blutspendezentrale.de/index.php?id=175>)

4.2 Chemically-defined serum-free media

Despite the disadvantages and critical ethical aspects discussed above, the global demand for FBS has increased continuously in the last decades. This phenomenon is not only driven by the growth of biotechnological and pharmaceutical industries, but is also associated with the current limitations of better defined media (van der Valk et al, 2010). Although increased selectivity for specific cell types or cell lines is one of the advantages of serum-free media, it also causes a decreased multiplicity in the use of the developed media. With almost exponentially increased number of newly identified or generated cell lines/types every year (Freshney, 2005), more cell-specific media are needed. Non-specific media often lead to slower growth compared to FBS containing media (Chowdhury et al., 2012; Gottipamula et al., 2014). Furthermore, the substitution of serum with defined components requires not only extensive knowhow and experience, but also a wide range of biochemical substances with high purity grade, the commercial availability of which is not always guaranteed or optimal.

One drawback for scientists moving away from FBS to chemically-defined serum-free media is the time and cost involved to identify a new FBS-free medium alternative for their specific cell types and to characterise cellular responses and “validate” their adaptation to the new environment. In other words: are the cells proliferating and generally behaving differently in the presence of FBS-free formulations? And if so, does that mean “incorrectly” or just “differently” from their behavior in the presence of FBS? The key aspect of cellular systems in the absence of FBS is to aid scientists to emulate human *in situ* environments in a robust and reproducible manner.

Modifying the medium to specific cell requirements can be costly as FBS-free options can be more expensive than FBS. Ethically concerned funding organisations are often happy to provide additional financial support to cover these additional expenses. However, it is important to appreciate that implementing a slightly more expensive cell culture based system may not only address the ethical concerns of bovine fetal blood collection, but also results in a cell based system that actually performs better (e.g., better reproducibility) and is able to better reflect the *in situ* human micro-environment.

Also, the increasing interest in primary cell culture is driving a shift in cell culture media preferences, with more scientists purchasing more chemically defined media (DePalma, 2016). The increasing demand for such media may promote a reduction of marketing costs over time. It should also be noticed that the costs associated with the use of FBS can be even higher, as FBS accounts for up to 85% of the overall cost of the medium, especially when calculated for large-scale cultures (Ozturk and Hu, 2005).

A commercial manufacturer is able to optimize the cost efficiency of the chemically-defined serum-free media not only with product specific know-how, but also by economically effective managed production. In the past decades, the reliable commercial supply of defined media and the standardization introduced by the bio-pharma industry have played important roles in the development of the animal cell culture technology and the application of serum-free media. Due to increasing regulatory pressure to eliminate animal components in cell culture products and biopharmaceuticals (EMA, 2013; ESAC, 2008; USP, 2012), increased demand for serum-free cell culture media has been observed. By 2022, a total value of 11.3 billion US dollars is estimated for the global cell culture market, with a cumulative annual growth rate (CAGR) of 7.1%. The cell culture media segment will have a leading share in the global market with the highest CAGR of 8.8% (Anon., 2015).

However, the commercialization of serum-free media is not without disadvantages. The exact formulations of many commercially available serum-free media are often a corporate secret. Alternative concepts, including hPL as alternative FBS substitutes, serum-free databases, and alternative cell culture concepts/systems need to be developed and existing platforms to be extended, to allow more widespread use of serum-free media also in countries where resources are limited.

Medium 199 (Morgan et al., 1950) and Ham’s Nutrient Mixture F-10 and F-12 (Ham, 1963, 1965) were the first culture media specifically designed for use without serum supplementation. Medium 199, in addition to the base components of electrolytes, amino acids and glucose, is highly enriched with vitamins and trace elements suitable for the serum-free culture of chicken embryo fibroblasts. Ham F-12 medium additionally contains zinc sulphate, putrescine, and linoleic acid. In this medium, CHO cells can be grown without serum supplementation.

Ham F-12, together with Dulbecco’s modified MEM (DMEM) in a 50:50 (v/v) mixture, is the basal medium for chemically defined serum-free cell culture (Barnes and Sato, 1980a, b). This basal medium is then supplemented with cell-specific hormones, growth factors, and trace elements. Furthermore, the addition of insulin, transferrin, and selenium (ITS) is required (Gstraunthaler, 2003; Jayme, 1999). Details are outlined in the report of the 2nd workshop (van der Valk et al., 2010), where a media pyramid was designed on the basis of current knowledge about serum-free media to inform on how to build a chemically-defined serum-free medium.

4.2.1 Development of new cell lines in serum-free medium only

The rapidly growing field of stem cell research, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), in particular, hold great promise for regenerative medicine, toxicology and biomedical research. Both hESCs and hiPSCs are generally maintained on inactivated mouse or human embryonic fibroblasts (feeder cells) or under feeder-free conditions (on extracellular matrices) in media supplemented with proprietary replacements as alternatives to FBS, in the effort to better control the phenotype of the cells (i.e., stem-ness maintenance, pluripotency, and differentiation towards a desired cell lineage). Indeed, given the presence of undefined or unknown components in FBS, serum-free culture systems now have been optimized to avoid the possible effects elicited by undefined differentiating growth factors and the risk of contaminations from pathogens potentially present in animal sera (e.g., mycoplasma, viruses, and prions) (Pistollato et al., 2012; Yamasaki et al., 2014).

These aspects were discussed in the first GCCP guideline, which dates back to 2005 (Coecke et al., 2005). A recently published document expands these aspects, specifically addressing which standards and quality controls should be considered for the culture of stem cell-derived models, in particular (Pamies et al., 2017). This publication, which represents a first step towards a revised GCCP 2.0, comments on the need to preferentially apply serum-free media, (animal) protein-free media and chemically-defined media, to avoid many disadvantages associated with the use of serum (Pamies et al., 2017). In this regard, as serum may induce spontaneous differentiation of iPSCs, fully chemically-defined media, supplemented with

appropriate hormones and growth factors (van der Valk et al., 2004, 2010) may be used. Serum-free media are thought to circumvent the batch-to-batch variability issues associated with serum, increase reproducibility and the differentiation efficiency of stem cells towards specific cell derivatives (Geraghty et al., 2014). Clearly, the use of alternatives to FBS has particular relevance when stem cells are intended to be applied for replacement therapies to avoid serum-related biosafety concerns (i.e., xenoimmunization, and the risk of disease transmission by pathogens) (Mannello and Tonti, 2007; Rakian et al., 2015; Schallmoser et al., 2008; Shahdadfar et al., 2005; Tonti and Mannello, 2008).

However, it should be noted that serum-free compositions may still require validation and monitoring similarly to serum-containing media. Particularly, serum-free media can also contain poorly defined supplements (e.g., pituitary extracts, chick embryo extracts, bovine milk fractions, bovine colostrum or platelet lysates [Chpt. 4.2, van der Valk et al, 2010]). For instance, B27 and the analogous NS21, which are commonly used supplements for the culture of neural cells, contain bovine serum albumin and transferrin, which can exhibit different batch-to-batch biological activity (Chen et al., 2008). Also, the so-called “basement membrane extract” (BME), purified from Engelbreth-Holm-Swarm murine sarcoma cells and marketed under different names (e.g., BME, Matrigel, and Cultrex) is prone to batch-to-batch variability. Additionally, while some supplement formulations are defined (e.g., Insulin-Transferrin-Selenium (ITS) supplement from Thermo Fisher), others are proprietary mixtures and, as a consequence, undefined, and for this reason different batches should be tested as for serum (Geraghty et al., 2014).

Likewise, other so-called “defined” media still contain complex plasma proteins. The widely used mTeSR™1 medium (STEMCELL Technologies Inc.) for serum-free culture of human embryonic (hESC) and induced pluripotent (hiPSC) stem cells is supplemented with human serum albumin, human holo-transferrin and human insulin (Ludwig et al., 2006). Optimization of mTeSR™1 led to the development of chemically defined albumin-free E8 medium (Chen et al., 2011).

A novel serum substitute, named XenoFree Serum Substitute (XFS2), specifically tailored to the needs of human cell culture, has been recently introduced by Celprogen, and appears to be suitable for the culture of cells with therapeutic potential. XFS2 was reported to support the culture of stem cells, progenitor cells, iPSCs, primary human cells, but also cancer stem cells, circulating tumor cells, and differentiated parental tumor cells. Interestingly, this serum replacement is completely composed of synthetic ingredients, supporting the culture of human cells under chemically defined condition (DePalma, 2016).

4.2.2 Case studies of chemically-defined serum-free applications

For several cell types, serum-free media have been described in literature and are now also commercially available, see for instance: ‘Serum-free media for cell culture’ compiled by the Animal Free Research UK (Animal Free Research UK, 2014). Below, selected examples of serum-free applications are reported.

4.2.3 Serum-free application of cell line L929

The mouse fibroblast cell line L929 was adapted to serum-free DME/F12+ITS medium (van der Valk et al., 2010). Before the adaptation, the cells were cultivated in standard cell culture flasks, fresh DMEM + 10% FBS medium was applied after 3 days and cells were split after 7 days. For the adaptation, 100,000 cells were transferred to a 25cm² Greiner Bio One Cellstar advanced (Frickenhansen/Germany) flask with 4 mL of serum-free medium and the culture routine was continued. As a result, the doubling time of the cells decreased from around 18 h to ca. 25 h. However, the serum-free cells proliferated over months and were available for experiments continuously during this time.

4.2.4 A test for eye irritation following The Cytosensor Microphysiometer (CM) Toxicity Test – INVITTOX n°130

To conduct tests for eye irritation of new chemicals, the IMOLA-IVD technology (Eggert et al., 2015) was adapted to perform similar experiments to the validated cytosensor microphysiometer test (INVITTOX no. 130²; Weiss et al., 2013). The IMOLA-IVD is a fully automated, label-free, microphysiological system mounted in an incubator to perform measurements at 37°C. The standard set-up consists of 6 IMOLA systems where BioChips with living cells are used. 6 fluidic modules with valves and air bubble detectors allow pre-programmed switching between flasks with cell culture media and the substance under investigation. A computer-controlled multi-channel pump drives the media to and from the BioChips. The BioChips are equipped with micro-sensors for pH, dissolved oxygen and impedance. Thus, it is possible to monitor the extracellular acidification, cellular respiration and changes in morphology of living cells. In the presented experiment, the system was programmed to supply L929 mouse fibroblast cells for 330 min with pure cell culture media. Then seven dilutions of sodium dodecyl sulphate (SDS) were subsequently pumped to the cells, starting with the highest dilution. During the experiment, the effect of seven dilutions of SDS toward extracellular acidification and impedance toward L929 cells was recorded. In a further development, the serum-free L929 cell line was used and the tests were performed with serum-free DME/F12+ITS medium (Wiest, 2017). Figure 2 shows the results of the chemically defined experiments. The first five recordings of the basal rates are shown, followed by seven dilutions of SDS. For better readability, the presented data was pre-processed using a script based algorithm to extract acidification rates and modulus of impedance (Wiest et al., 2016) from the raw data.

The results show that a test for eye irritation with L929 cells cultivated in serum-free medium is possible.

² INVITTOX no. 130. (<https://eurl-ecvam.jrc.ec.europa.eu/validation-regulatory-acceptance/topical-toxicity/eye-irritation/>) Accessed: 02/21/2017

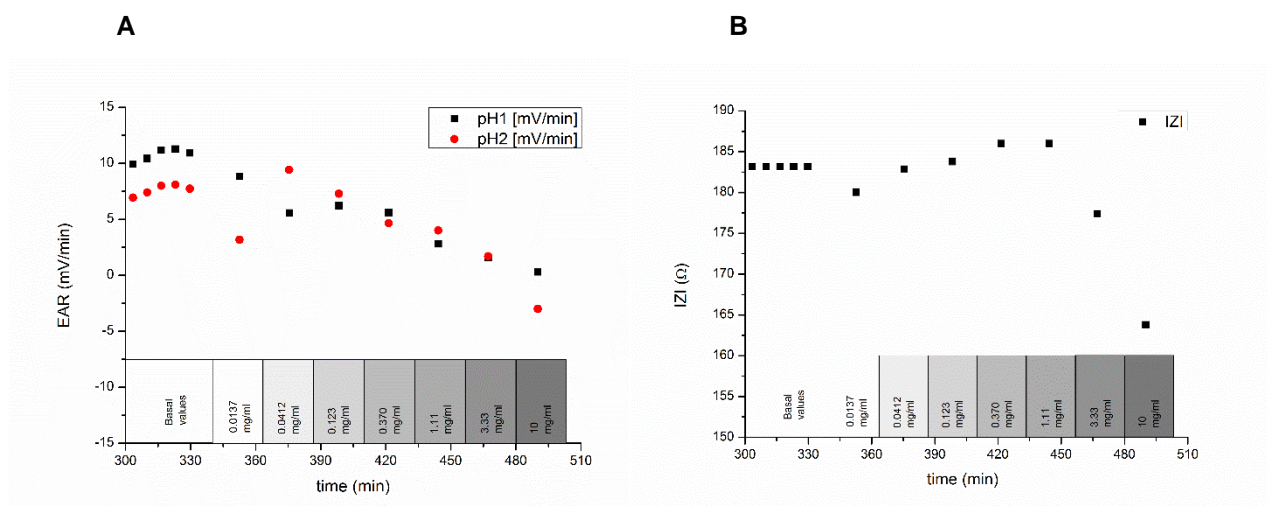


Fig. 2: Test for the eye irritation potential of seven dilutions of sodium dodecyl sulphate toward L929 fibroblasts
A: extracellular acidification rate (EAR), B: modulus of impedance (IZI)). The first five recordings of the basal rates are shown, followed by seven dilutions of sodium dodecyl sulphate.

4.2.5 A test for cytotoxicity following ISO 10993-5

Tests for biocompatibility/cytotoxicity of new materials are needed for biological evaluation of medical devices as described in ISO 10993-5 (ISO, 2017). Besides extract tests and indirect contact tests a direct contact test is specified, which requires the incubation of mammalian cells in contact with a device. The norm specifies, among others, positive, negative and a blank control, a minimum of three replicates, sterility and the use of established cell lines. For the test, the serum-free L929 cell line was used and as negative controls silicone or aluminium were suitable. For the positive controls, PATTEX Multi or a copper band is recommended. For validation, a number of experiments were performed in parallel with L929 cultured in DMEM + 10% FBS. The serum-free tests delivered the same results as the FBS based tests.

In conclusion, a test for cytotoxicity according to ISO 10993-5 is possible with L929 fibroblasts in chemically defined cell culture medium (Wiest, 2016).

4.2.6 Organ system

A 4-organ system has been fabricated consisting of liver, cardiac, neuronal and muscle tissues in a serum-free, circulating medium for 14 days that was used to determine multi-organ toxicity for 5 common drugs (Oleaga et al., 2016). This was accomplished utilizing a pumpless platform as shown in Figure 3.

Computer simulations of the platform utilizing a commercially available computational fluid dynamics package (CFD-ACE+), established flow rates and resultant shear stress within physiological ranges. Viability of the system for 14 days was demonstrated with functional activity of the human cardiac, muscle, neuronal and liver modules. The integrated modules were evaluated for their response to 5 drugs at 7 days after a 48 hour treatment regime. System evaluations were based on cellular viability, production of urea and albumin, electrophysiological responses and cardiomyocyte and muscle contraction frequency. The drugs doxorubicin, atorvastatin, valproic acid, acetaminophen and N-acetyl-m-aminophenol were tested and the results correlated well with toxicological effects reported in the literature (Oleaga et al., 2016). This low cost platform was self-contained and allowed for continuous serum-free medium circulation with periodic medium supplementation and analyte analysis. The operating time has been extended to 28 days with daily removal of 30% of the reservoir volume for analysis and replacement with fresh medium. This system is the first microfluidic model to demonstrate the capacity to promote the survival and functional competence of 4 interconnected tissue modules in serum-free medium over extended culture periods. The prototype model used human cells: (a) for the cardiac compartment induced pluripotent stem cells (iPSC) differentiated into cardiomyocytes (from Cellular Dynamics International, CDI) were applied; (b) for the skeletal muscle, compartment skeletal myofibers were prepared by isolation and differentiation steps described in Guo et al. (2014); (c) for the neuronal system, motoneurons were differentiated (Guo et al., 2011) from a previously isolated human spinal cord stem cell (hSCSC) line; and (d) for the liver compartment, a cell line from a hepatocellular carcinoma HepG2/C3A (ATCC® CRL-10741) was used in the prototype system. The serum-free medium is based on that described in Guo et al (Guo et al., 2011) except for the addition of an antibiotic/antimycotic cocktail and the elimination of the G5 supplement. This system demonstrated that complex human *in vitro* systems can be constructed with a common serum-free medium without FBS.

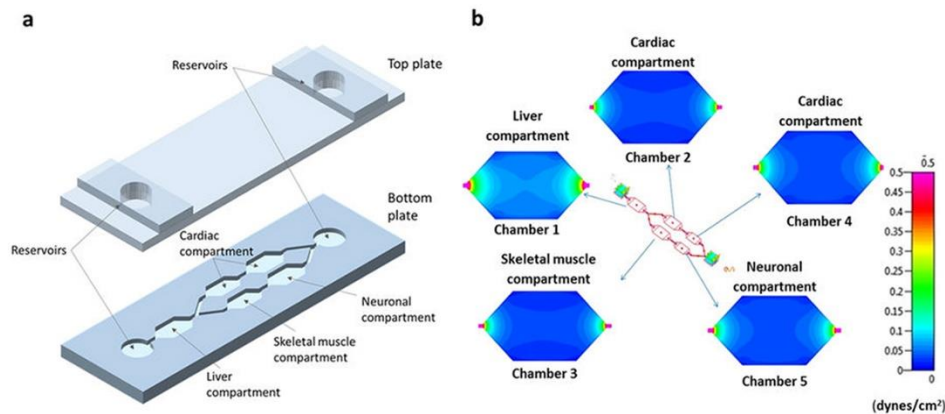


Fig. 3: Schematic view of a 4-organ microfluidic platform

(a) Schematic view of the microfluidic platform showing the different cell compartments of a 4-organ system. The system contained two holders for the separate culture devices. Total fluid volume was approximately 4 mL between the chambers and reservoirs. The sizes of the culture compartments were 35.8 x 18.4 x 0.3 mm for Chambers 1, 2, 3 and 29.8 x 15.4 x 0.7 mm for Chambers 4 and 5. The connecting channel dimensions were 5.7 x 1 x 0.3 mm. (b) Shear stress distribution in each compartment of the system. Reproduced with permission from (Oleaga et al., 2016).

5 Serum-free databases

As discussed in the preceding text, a broad array of serum-free culture media has been published in the past decades. Unfortunately, serum-free medium formulations have in most part been confined to specific cell types or cell lines, but their application is now being extended to a broader list of cell types. However, the search for cell-specific serum-free media can be time-consuming and tedious work requiring either a broad literature survey or a search for commercially available serum-free media (Brunner et al., 2010).

A solution to this issue is the development of freely accessible serum-free media databases with comprehensive search functions. These databases should be regularly updated and their existence promoted. This approach also addresses the continued issue of “raising awareness”, is cost effective and a relatively simple activity to significantly and meaningfully help to reduce or even completely replace FBS in cell culture medium wherever possible.

A decade ago, Focus on Alternatives (FoA), a like-minded group of charities and organisations³, collaborated to produce an excellent and impactful resource to further increase the awareness of commercially available animal-sera free cell culture media. Animal Free Research UK (AFR), decided to independently continue to support and develop this important database for the wider scientific community⁴. Whilst there are not FBS-free alternatives for every conceivable application, there is great merit in helping to address the continuing “awareness” issues.

Currently available FBS-free media databases (e.g. the one hosted and curated by the AFR) are limited in that they typically only promote commercially available alternatives and do not contain details of media described in the wider scientific literature. For many cell types, serum-free media are already commercially available. These FBS-free media alternatives are generally cell type specific (Tan et al., 2015). But, unfortunately, not for all cell types have yet serum-free media been developed. Additionally, for some cell types there are a number of viable options, however, there is a limitation in that the different options can elucidate different cellular responses (Rodrigues et al., 2012). Although these media are widely regarded as chemically-defined, the formulations of such alternative solutions are seldom released, which the authors assume is for proprietary reasons. As a general rule, limited information is usually communicated (e.g., whether the medium contains animal-derived components or not). In some situations, knowledge of the precise formulation of the medium may assist in developing a robust and critical interpretation of experimental results. This information may also provide opportunities to improve the medium to obtain the expected (*in situ*) properties of the cell. It is therefore important that any future database does not ignore the FBS-free media solutions as described in the literature. However, to date, this information has not been collated. To facilitate the identification of existing serum-free media for a specific cell type, and to provide the digital infrastructure for scientists to share experiences as well as best practice and media improvements, it is recommended that any future database on FBS alternatives also includes published serum-free media formulations.

Any such database should incorporate a number of key features, i.e. it should be:

- Free to use
- Searchable under numerous different fields
- Hosted through reliable and well respected digital avenues (ideally with duplicate sites)

³ Dr Hadwen Trust (now: Animal Free Research UK), FRAME, The Humane Research Trust, RSPCA, Advocates for Animals, UK Human Tissue Bank and the Lord Dowding Fund.

⁴ From August 2017, the database will be available online (www.serum-free.org) as part of the 3Rs Database program of the 3Rs-Centre Utrecht Life Sciences.

- Continually curated and updated (and dated to demonstrate this)
- Populated, not only with commercially available options but also including successful, peer-reviewed published solutions and their methods.
- Well publicised through appropriate sources for maximum impact throughout the scientific community (e.g., conferences, webinars, social media – particularly LinkedIn-dedicated groups, intranet channels, organisations newsletters and through targeted advertising campaigns in scientific journals and 3Rs focussed websites).

To achieve these outcomes, it is vitally important to establish secure and sustainable funding sources.

6 Conclusions and recommendations

From an animal welfare point of view the current practice of obtaining FBS is unacceptable because of the potential suffering the fetuses might experience during the entire process. A compulsory rule for the stunning or killing of fetal calves before blood collection should be the immediate measure to reduce the suffering of unborn calves for science and commercial activities.

As indicated earlier in the manuscript, when FBS is harvested from living fetuses in Europe it has to be considered a procedure under the Directive 2010/63/EU and therefore, if not banned, should at least undergo the proper peer review and authorization process.

In the meantime, there is need for better legal protection of all fetal forms of vertebrates as we cannot exclude suffering before birth. Generally, authorities, at least within the EU and other countries that legally protect fetuses under their animal experimentation legislation, will have to find ways to assure that all provisions are fully applied to FBS production, most prominently a cost-benefit analysis and rigid application of all 3Rs. As mentioned before, this might not be possible to implement, whereupon the final consequence would be that as alternatives are developed, no licensing of FBS production should be issued if equivalence is demonstrated (ESAC, 2008).

Not only because of this should the scientific community go one step further and question the frequent and routine use of FBS in scientific projects from a moral and legal perspective. Even without – at present – proper legal handling of the issue, it seems evident that there is a moral obligation to apply the 3Rs principle to the use of FBS.

The obligation to use alternative methods wherever possible is very clear and it is therefore even less understandable that the 3Rs continue to be ignored in this matter, as scientists around the world appear to be hesitant to switch to serum-free media for their experiments. Scientists who develop 3Rs methods especially should promote serum-free methods, independent of legal pressure.

In addition to the ethical and legal aspects there are several scientific aspects of using FBS of which we conclude the following.

There is a clear need for the replacement of FBS and for the design of serum-free cell culture media, respectively, which will result in better standardization and data reproducibility of *in vitro*-applications (Gstraunthaler et al., 2014), and will substantially contribute to GCCP (Coecke et al., 2005). Due to the large commercial interest in providing FBS, fraudulent practices have become apparent, which will have an effect on, among other issues, reproducibility, adding to the reproducibility crisis (Baker, 2016a). The use of FBS in Biological Resource Centres is also becoming problematic since changes in the phenotype and genotype of cell lines when changing to other batches of serum might not always be obvious.

Although FBS is a powerful universal supplement for the cell culture, from a scientific point of view FBS is not suitable for the following applications:

- (1) To study the functional or regulatory effects of exosome, growth factors or hormones due to the contamination caused by serum (Dietrich et al., 2011; Rauschenberger et al., 2016);
- (2) To induce the differentiation of stem cells in a controlled or reproducible manner (Puts et al., 2016);
- (3) To perform co-culture tests with fibroblasts (Harati et al., 2016; Nikolakis et al., 2015).

Furthermore, it is highly recommended to replace FBS with chemically defined components, particularly in *in vitro* toxicity tests, to improve the sensitivity and reproducibility (Hahne and Reichl, 2011; Peter et al., 2007), since serum free formulations may still contain poorly defined supplements. Unfortunately, no universal chemically defined medium is yet available, meaning that for every existing cell line the chemically defined medium has to be developed. To facilitate the identification of existing serum free media it is recommended that freely available database are established, providing information both on commercially available formulations as well as those described in literature.

Although the workshop and its conclusions focussed mainly on the use of FBS in research, also in the area of cellular therapies (Karnieli et al., 2017) and *in vitro* meat production (Post, 2012) the same issues apply.

However, despite the severe ethical and scientific concerns, a stop in the use of FBS in cell cultivation cannot be realized without offering real technical alternatives. Many of the commercial available chemically defined FBS replacements or serum-free media are still to be improved with respect to the performance and the cost, compared to FBS. To effectively reduce the presence of FBS, hPL can be considered as a good alternative to serum according to its advantages mentioned above, however, with limited potential to develop fully defined media. Alternatively, serum replacements can be used to significantly reduce the amount of FBS in the cell culture media. For most of the commonly used cell lines, cultivation of the cells only becomes challenging if the final concentration of FBS is lower than 1% (in combination with additional chemically defined serum-free replacements). At this low concentration, the culture medium has become notably defined, while the reproducibility of the experiments can be improved significantly (Schlingensiepen et al., 2011). However, the examples presented in chapter 5 demonstrate that it is possible to obtain reliable results in serum free cell and complex organ systems.

Furthermore, when establishing new cell lines, it is recommended to start from the beginning with serum-free, preferably chemically defined, media.

Anyhow, the use of FBS, when collected in a way that causes suffering, seems at odds with, and morally problematic in the context of, application or development of *in vitro* methods that are meant to replace and reduce animal use⁵.

For all these reasons, in the short term, serious effort should be made to support the production and marketing of both traditional and more recently generated cell cultures, all under serum-free conditions, and encourage the scientific community to use them. Fortunately, guidelines such as the Good Cell Culture Practices discourage the use of FBS (Coecke et al., 2005; Pamies et al., 2017). In addition, because of contamination issues, the use of FBS during the production of biologicals is discouraged (EMA, 2013; USP, 2012).

In line with this, we propose a list of recommendations with the aim to ease and support this transition process (Table 2).

Tab. 2: List of recommendations to ease and support the transition toward serum-free culture conditions

Recommendations		Comments
R1	Funding for the production and marketing of serum-free culture models should be generally supported and implemented in Europe and worldwide	The use of FBS currently represents a potentially legal issue (as per the Directive No 2010/63/EU); also, at initial production and marketing stages, chemically defined media can be expensive
R2	Funding should be allotted for research proposals aiming at developing cocktails of defined growth factors, hormones etc., suitable to support <i>in vitro</i> cell growth, proliferation, differentiation and terminal maturation of different cell types, followed by formal validation of serum-free culture models	National funding programs should require that any institution supporting 3R methods should prioritize for funding grant proposals based on serum-free methods/models; funding bodies should give the proponents enough time to work on the shift to serum-free conditions, when intentioned to do so
R3	There should not be the need to keep referring to FBS-based protocols, provided that a new serum-free method is available and equally comparable	This may encourage scientists to switch to serum-free approaches, avoiding the concern of losing previously generated data
R4	Commercially available serum-free supplements should be standardized and rigorously validated for various types of cell cultures	This would help circumvent batch-to-batch variability, as it commonly happens with FBS
R5	Cell suppliers, such as stem cell banks, should make specific requirements in culture method descriptions, addressing the need to apply FBS-free media (if such exists) particularly for the culture of (pluripotent) stem cells	Cell banks should prioritize cell culture methods that apply serum-free protocols, to better control cell phenotype, and make more efforts to advertise cell cultures that do not require the use of FBS
R6	Serum-free databases, with comprehensive search functions and free access, should be implemented	These represent important tools to support researchers with information on the selection of appropriate serum-free media; commercial records should be expanded with newly published information
R7	Educational and outreach activities should be organized to inform researchers on the ethical and scientific limitations of FBS, current regulations, and commercially available serum-free media	These activities may be organized in academic institutions and by using webinars, lectures, LinkedIn groups and intranet channels
R8	A legal framework and guidance for authorities licensing procedures under Directive No 2010/63/EU is needed for the production of FBS if carried out on EU territory	Report could be send to DG ENV and this specific issue mentioned in the cover letter.

⁵ "Article 4

Principle of replacement, reduction and refinement

1. Member States shall ensure that, wherever possible, a scientifically satisfactory method or testing strategy, not entailing the use of live animals, shall be used instead of a procedure."
(Dir. 2010/63/EU)

7 References

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Conflict of interest statement

J. Wiest is shareholder and CEO of cellasys GmbH.

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