Review Article

Case Studies Exemplifying the Transition to Animal Component-free Cell Culture

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Abstract

Cell culture techniques are strongly connected with modern scientific laboratories and production facilities. Thus, choosing the most suitable medium for the cells involved is vital, not only directly to optimise cell viability but also indirectly to maximise the reliability of the experiments performed with the cells. Fetal bovine or calf serum (FBS or FCS, respectively) is the most commonly used cell culture medium supplement, providing various nutritional factors and macromolecules essential for cell growth. Yet, the use of FBS encompasses a number of disadvantages. Scientifically, one of the most severe disadvantages is the lot-to-lot variability of animal sera that hampers reproducibility. Therefore, transitioning from the use of these ill-defined, component-variable, inconsistent, xenogenic, ethically questionable and even potentially infectious media supplements, is key to achieving better data reproducibility and thus better science. To demonstrate that the transition to animal component-free cell culture is possible and achievable, we highlight three different scenarios and provide some case studies of each, namely: i) the adaptation of single cell lines to animal component-free culture conditions by the replacement of FBS and trypsin; ii) the adaptation of multicellular models to FBS-free conditions; and (iii) the replacement of FBS with human platelet lysate (hPL) for the generation of primary stem/stromal cell cultures for clinical purposes. By highlighting these examples, we aim to foster and support the global movement towards more consistent science and provide evidence that it is indeed possible to step out of the currently smouldering scientific reproducibility crisis.

Keywords

animal component-free, cell cultures, chemically defined medium, FBS-free, fetal bovine serum, human platelet lysate, multicellular models, reproducibility crisis, transition, xeno-free

Introduction

Cell cultures are widely used as models for biological processes in whole organisms. They are far easier to use, access and manipulate than model organisms or even humans themselves. Thus, the use of cell culture technologies has impacted the whole field of biomedicine, ranging from basic biological and medical research right through to drug testing (efficacy and toxicity), the manufacture of antibodies, biopharmaceuticals and vaccines, assisted reproductive technology and cell therapy, regenerative medicine and tissue engineering.

Typically, cell cultures are considered to be reproducible, traceable and transferable, at least when compared to whole animal models — in which intrinsic complexity, heterogeneity and inter-species differences are quietly overlooked. Yet, it is increasingly obvious that studies based on the use of cell cultures can also suffer from reproducibility issues. We are convinced that this is largely related to the reagents and materials used in the cell culture process, which are frequently undefined and not standardised.

Cell culture medium is the most important factor for successful cell culture technologies, as it permits the modulation of cellular fitness and function. Since the beginning of routine cell culture in the 1880s, cell culture

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CORRECTION (November 2022): Article updated online to remove "accutase" from non-animal replacements in the "Xeno-free aspects beyond fetal bovine serum" section.

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technologies have developed tremendously.[1] However, the process of cell culture plays a significant role in the reproducibility crisis in the biomedical sciences,[2,3] particularly when ill-defined supplements, such as serum, are employed. Baker et al.[2,3] highlighted that numerous variables influence the outcome of cell culture-based experiments, with differences between the serum batches representing a major source of variability. Fetal bovine serum (FBS; also known as fetal calf serum, FCS) remains the most commonly used cell culture supplement, given that it exerts numerous favourable effects on *ex vivo* and *in vitro* cell and tissue cultures. For example, FBS mediates cell adhesion and cell–cell interactions, as well as promoting cell proliferation, survival, differentiation and senescence.

In addition, many other cell culture components — such as bovine pituitary extract, matrix molecules (e.g. Matrigel™), adhesion and growth factors, carrier molecules (such as albumin), as well as various enzymes used to extract cells from tissues and disrupt culture monolayers (e.g. trypsin) — are animal-derived. Ethical issues with animal-derived components, especially FBS, have been discussed for years[4–11] and still continue to spark debate.[12,13] Techniques that aim to replace procedures on live animals should be animal component-free, in order to avoid collateral animal suffering. As highlighted below, non-animal derived replacements to many of these components are already available, and their use will help circumvent the various drawbacks of using animal-derived substances. Besides the ethical and animal welfare aspects, batch-to-batch variability, ill-defined composition, and the risk of pathogen transmission or an immune system reaction, all call for a transition to animal component-free methods.[14,15]

Several factors influencing the successful transition to animal component-free culture

Different types of serum replacements are available. Karnieli et al.[16] suggested a consensus nomenclature for serum replacements and serum-free media. They recommended classifying media into: serum-based; serum-free; xeno-free; animal component-free; protein-free; and chemically defined. It appears obvious that a chemically defined medium, avoiding the use of any animal-derived substance, would be desirable for various reasons. Increased reproducibility of the data generated may be a valuable scientific argument,[8,17] but the transition to fully chemically defined media is currently not yet possible for every cell type and cell line. Human platelet lysate (hPL) has been introduced with a view to replacing FBS, especially for the expansion of human cells for clinical use in cell therapy or tissue engineering.[11,18,19] In addition, human serum has been similarly used in the cellular therapy field.[20] However, human-derived reagents such as hPL and human serum need to be critically discussed as well.[21] Platelet concentrates for clinical use in humans have a shelf-life of between three and seven days at room temperature,[22] routinely resulting in their disposal when this shelf-life has expired.[23] To avoid potential competitive situations relating to the use of these valuable resources, we suggest that platelet concentrates should only be used for hPL production when they are past their expiry date for clinical use.

Selection and optimisation of the animal component-free medium. As mentioned, cell culture media are often immensely complex with regard to their final composition. Burgener and Butler[24] wrote a recommended chapter on cell culture media, which includes a description of their history and strategies for the development of serum-free media. The transition to animal component-free conditions may require a series of optimisation steps. The first step is to find out whether animal component-free media are available, and have already been evaluated, for the respective cell line. The Fetal Calf Serum-Free Database (https://fcs-free.org/) is a good starting point, as it lists commercially available serum-free media and summarises medium compositions from the scientific literature for various cell types. Commercially available chemically defined media may require optimisation for the specific cell type and experimental approach. When transitioning to defined media, studies are often planned to screen the most efficient compounds and then define the optimal concentrations and combinations. Such methodology permits the testing of various compounds simultaneously, and the determination of potential interactions, in addition to their individual effects.[25–27] This approach is helpful to study the influence of different components of a cell culture medium on cell viability without testing all possible combinations. However, it has to be noted that it should only be used for independent variables, i.e. if the different components of the cell culture medium may interact, the design does not deliver clear-cut results.

Impact of the culture vessel material. An important part of cell culture that has not been extensively discussed refers to the chemistry of the plastics used for tissue culture (i.e. the plates and flasks), which may have a significant impact on the successful transition to serum-free medium. Tissue culture vessels are most commonly made of polystyrene, which is naturally hydrophobic.[28] The easiest way to turn polystyrene hydrophilic is by plasma gas treatment, which introduces oxygen in the styrene molecules, which renders the surface negatively charged when in contact with water. This is an extremely simple surface treatment, the simplicity of which has been shadowed by the use of serum containing a complex blend of attachment proteins and other proteins, that form a ‘cover’ over the unnatural surface. A switch to defined medium may generate greater concern over the

chemistry of the cell culture vessel plastic, as it could possibly require the introduction of both positive and negative charges by the manufacturers.

Control of the culture medium pH. Of equal importance with regard to the successful transition to serum-free medium is the tight control of medium pH during cell growth. Media are, in general, buffered by bicarbonate in equilibrium with the surrounding CO_2 present in the incubator atmosphere. To maintain correct physiological pH, the CO_2 in the incubator needs to be kept at 5–10%, depending on the level of bicarbonate in the medium. The culture medium is usually stored in a bottle at 4°C between uses. As the liquid in the bottle is progressively used, the amount of atmospheric air, at a CO_2 level of 0.04%, will increase. This will result in an increasingly alkaline medium. FBS and other sera protect cells from the toxic action of an alkaline medium, until such time as the correct pH can be established, when the cultures are transferred to the CO_2 incubator. To prevent this change in pH, many media are supplemented with the zwitterionic buffering agent HEPES (hydroxyethylpiperazine ethane sulphonic acid). However, HEPES is xenobiotic — i.e. a synthetic chemical that is foreign to the cells — and, as such, its use should be avoided whenever possible. In addition, light-exposed medium containing HEPES can become cytotoxic due to formation of hydrogen peroxide.[29] The gas exchange in the medium that leads to alkalinity can be overcome with certain measures, such as replacing the atmosphere in the medium bottle with air containing 5% CO_2 (by gassing the bottle or by always pre-equilibrating the medium to be used in the incubator).

These factors illustrate the challenges that need to be faced when transitioning to animal component-free media, which will in turn lead to more defined experimental conditions, more reliable results, and more reproducible science. Even though, at first glance, it might appear to be a complex and challenging task, implementing these measures will improve basic research and drug development, and eventually lead to better outcomes for scientific progress and for public health alike, and help to restore faith in the validity of scientific discoveries.

Xeno-free aspects beyond fetal bovine serum

To establish a completely xeno-free research environment, other aspects need to be considered in daily laboratory practice, in addition to the replacement of FBS. The passaging of cells is usually performed with the help of a cell dispersion enzyme, which may be of animal origin. In this situation, the user can choose from a variety of non-animal replacements such as TrypLE™ Express, papain or others. Another issue to consider is the provision of an extracellular matrix (ECM): culture vessels, Transwell® inserts, etc., can be coated with mouse tumour-derived Matrigel™, or with other animal-derived components such as fibronectin, collagen or laminin. These can be replaced with human recombinant solutions, synthetic chemicals, or even by simple chemical treatment of the plastic prior to use.[30] To further improve reproducibility, all standard protocols used in the laboratory (e.g. for the freezing and thawing of cells, cell counting, etc.) should be reviewed and modified as necessary, to transition to the use of chemically defined and xeno-free substances. Further guidance can be found in the review by Yao and Asayama[31] and in the Good Cell and Tissue Culture Practice 2.0 guidance document.[32]

Despite the importance of these other products and protocols in terms of their need for replacement, within this brief report we will solely focus our discussion on the replacement of FBS. We highlight three different scenarios and provide some case studies of each, namely: i) the adaptation of single cell lines to animal component-free culture conditions by the replacement of FBS and trypsin; ii) the adaptation of multicellular models to FBS-free conditions; and iii) the replacement of FBS with hPL for the generation of primary stem/stromal cell cultures for clinical purposes.

Adaptation of single cell lines to animal component-free culture conditions

In our first scenario, we look at examples of successful adaptation of a cell line from growth in FBS-supplemented medium to an animal component-free culture. In general, two procedures can be used to adapt cells to serum-free conditions — either a direct switch from FBS-containing to serum-free medium, or a sequential adaptation by successively decreasing the concentration of serum-containing medium over time, relative to serum-free medium (i.e. 100%, 75%, 50%, then 25% serum-containing medium, and then finally 100% serum-free medium).[33]

With regard to the vast array of cell types that are available, the use of human cells instead of animal-derived cell lines is much preferred, in order to increase transferability of the experimental data to human conditions. However, there are abundant examples of non-human cells that have long been established in the scientific community, some of which are mentioned below. These include: murine L929 cells; renal epithelial cells of the African green monkey (Vero cells); and Chinese hamster ovary (CHO) cells.[34]

An important distinction must be recognised between primary cell cultures/finite cell lines (which have to be re-established repeatedly *ex vivo*), and immortalised cell lines (which represent cells that have been cultured *in vitro* for decades). For example, strain L was one of the first cell strains to be established in continuous culture and was

derived from a mouse in 1940.[35] Its clone 929, better known as L929, was developed back in 1948[36] and has been in use since then. It is, as expected, a very well characterised cell line.

An animal cell line *per se* is not animal component-free. As such, from an animal welfare point of view, the production of *new* animal-derived immortalised cell lines would hardly be considered acceptable, especially when harvested invasively. However, animal suffering could also be involved when cells are harvested non-invasively, e.g. from animals held in intensive husbandry. Nevertheless, the use of long-term established animal-derived cell lines is not considered to be as problematic, but rather beneficial in terms of sustainability and data reproducibility. However, to obtain optimal transferability of *in vitro* data to the human situation, human cells will always represent the best model.

Example 1: Vero cells in human vaccine production

According to Kiesslich and Kamen, serum-free medium is "preferred by vaccine manufacturers due to regulatory concerns regarding the risk of contamination".[37] To facilitate production scale-up, Rourou et al.[38] reported how they adapted adherent-growing Vero cells (a non-human cell line) to suspension growth in different serum-free media. The authors compared three different protocols, in which the Vero cells were either (first) adapted sequentially to serum-free conditions and then to suspension culture, or (second) *vice versa*, or (third) adapted to a parallel combination of serum-free conditions and suspension culture. The highest cell numbers were obtained by adapting to serum-free medium over two passages, and then adapting the cells to suspension culture. It was found that two commercially available serum-free and chemically defined media performed similarly to the serum-containing medium.

Example 2: Biobanked cell lines

Fusi and Dotti recently reported the sequential adaptation of the human epithelial carcinoma cell line HEp-2 to serum-free medium.[39] They compared sequential adaptation to directly switching the cells to a medium supplemented with 5% hPL. Following the successful adaptation to animal serum-free culture, they then evaluated the replacement of animal trypsin with a non-animal recombinant protease (TrypLE™ Express). They employed real-time analysis to determine and compare growth curves, leading them to conclude that the HEp-2 cell line could be adapted to culture methods based on totally animal-free products that were as effective as FBS and trypsin. This was the starting point for the transition of further biobanked cell lines to animal-free culture conditions.

Example 3: Real-time tools

The development of optimal FBS-free cell culture medium by using conventional techniques takes months rather than weeks. To save resources and time, real-time approaches can eliminate unsuitable medium candidates within hours. The cellasys #8 test is an automated microphysiometric system[40] that can supply the cell line under analysis with two different cell culture media and then measure any changes in cellular metabolism and cellular morphology within minutes. Thus, it can help identify an optimised FBS-free cell culture medium often within 24 hours.

Figure 1 presents an illustration of the cellasys #8 test, which is executed with the six channel-intelligent mobile lab for *in vitro* diagnostic (6 × IMOLA-IVD) system. The cell line under analysis is first supplied for 6 hours with FBS-containing medium (reference medium), followed by 6 hours with an FBS-free chemically defined medium (test medium), then 4 hours with FBS-containing medium and 4 hours with the FBS-free chemically defined medium. Finally, a sodium dodecyl sulphate (SDS)-containing medium is fed through the system for 4 hours to lyse the cells. Every 10 minutes, changes in cellular metabolism and morphology are monitored. Besides the effects of the different cell culture media in the 'Treatment I' phase, it is also useful to distinguish inhibitory toxic effects in the 'Recovery' phase.[41]

A combination of real-time analysis and Plackett–Burman experimental design has been presented by Zhao et al.[27] as a high-throughput strategy to screen a serum free medium to support the growth of PC-3 prostate cancer cells. Schmidt et al.[42] employed a three-stage approach, encompassing the cellasys IMOLA-IVD system, to assess the impact of a chemically defined medium on CaCo-2 cells. The study showed successful long-term culture (> 100 days), as well as differentiation towards the formation of microvilli of the CaCo-2 cells. The simple, chemically defined medium used by Schmidt et al. consisted of a 1:1 mixture of DMEM and Ham's F12, with the addition of 14.7 mM NaCl, 20.9 mM $NaCHO_3$ and 5 ml/L ITS.[43]

Adaptation of multicellular models to fetal bovine serum-free conditions

Example 1: 'Body-on-a-chip' model

In 1995, the group of James J. Hickman reported a serum-free, defined culture system for neurons.[44] They successfully propagated various cell types in culture for several months, including human neurons, glia, muscle and cardiomyocytes. By using the same basic serum-free medium, they developed a functional neuromuscular junction model.[45] This methodology was later extended to

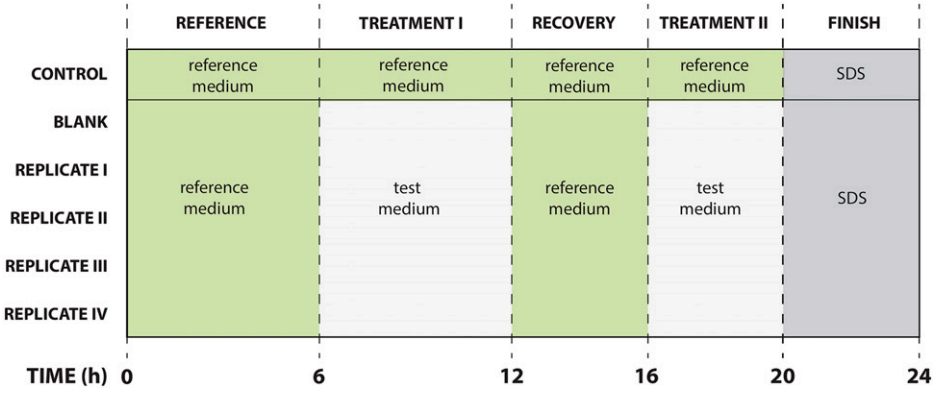

Figure 1. The cellasys #8 test scheme for the evaluation of an FBS-free chemically defined medium *versus* FBS-containing medium. The 'reference medium' is the FBS-containing medium; the 'test medium' is the FBS-free chemically defined medium under evaluation; 'SDS medium' is for instigating cell lysis. CONTROL channel = cells supplied with FBS-containing medium only; BLANK channel = no cells (media only); REPLICATE I–REPLICATE IV = channels contain the cells being tested (in quadruplicate). At each step, changes in cellular metabolism and cellular morphology are monitored. FBS = fetal bovine serum; SDS = sodium dodecyl sulphate.

various multicellular 'body-on-a-chip' models.[46] A human adipose-liver-on-a-chip model that permits the study of factors involved in non-alcoholic fatty liver disease (NAFLD) is a recent example of the use of defined media in microphysiological systems.[47] For the development of this model, patient-derived hepatocytes initially cultured in FBS-containing medium were switched to serum-free conditions on day 3. Adipocytes were differentiated from preadipocytes, and then cultured singly or in combination with the hepatocytes in a two-chamber culture housing system. Different serum-free media compositions mimicking NAFLD were applied. The platform facilitated the monitoring of physiological responses to metformin treatment, indicating that this system might be useful for the testing of novel drugs to treat NAFLD.

Example 2: Vascularised 3-D tissue constructs

Organotypic vascular cells, particularly organ-specific endothelial cells, control vascular niche functions during organ development, and under physiological and pathophysiological conditions. Cultured adult endothelial cells, however, lose this organotypic vascularisation capacity. Palikugi et al.[48] recently reported the serum-free culture of "adaptable haemodynamic endothelial cells for organogenesis and tumorigenesis". Mature endothelial cells could be 'reset' by transient reactivation of the transcription factor ETV2 (ETS variant transcription factor 2). These cells — cultured in a serum-free medium with knockout serum replacement medium and cytokines on a serum-free 3-D matrix — self-assembled to 3-D vessels and were capable of, for instance, vascularising human islets in a microfluidic device *in vitro*. Likewise, 3-D vascularised tissue constructs were bio-fabricated from embryonic stem cell-derived endothelial cells and pericytes under chemically defined conditions in a fibrin matrix.[49] A vascular network formed within nine days and remained stable for three weeks, exerting barrier function and responding to histamine.

The use of human platelet lysate in the isolation and expansion of primary human cells for cell therapy

Example 1: Human mesenchymal stromal/stem cells

Human mesenchymal stem/stromal cells (hMSCs) are widely used in cell therapy.[50,51] Around 65–80% of listed hMSC-based clinical trials in recent years utilised FBS in the isolation and expansion of the hMSC.[19,52] However, initial concerns about pathogen transmission and the risk of an immune system reaction prompted the move towards FBS-free culture conditions.[53] Human platelet lysate (hPL) has been identified as a possible FBS replacement. It is rich in human growth factors and produced in most cases from platelets/platelet concentrates past their expiry date for human clinical use.[54]

Protocols were developed to isolate and expand hMSCs in human hPL or human serum, respectively, and the cell functionality compared to that obtained with cells cultured in FBS-supplemented medium.[18,55] The most obvious finding was that the human-derived supplements accelerated the proliferation of hMSCs, compared to the same concentration of FBS, without affecting their replicative ageing and inducing cellular transformations.[56] Key MSC functions, which render them interesting for clinical applications, were fully maintained.[11] The most important findings were some evident changes in gene expression and functional characteristics that could potentially affect mechanisms of action in certain clinical settings, which

were noticed in cells cultured in FBS, but not in those cultured in hPL or human serum.[57–59] Probably, the fetal factors within FBS may have caused a dedifferentiation of cells. This may also explain the observation that high concentrations of FBS facilitate the reprogramming of adipose-derived MSCs to induced pluripotent stem cells.[60]

Example 2: Human CAR T-cells

Based on the successful use of hPL for the commercial production of clinical grade MSCs, hPL has also been tested in commercial methods for immune cell generation, e.g. macrophages, dendritic cells and CAR (chimeric antigen receptor) T-cells. Torres Chavez et al.[61] observed that CAR T-cells expanded in hPL, compared to cells expanded either in FBS or in human serum, exhibited a naïve-like and central memory T-cell phenotype, accompanied by superior proliferation, long-term persistence and anti-tumour functionality upon adoptive transfer. Similar findings were reported by Canstrani et al.,[62] who demonstrated that hPL improved lentiviral transduction and enriched T-cells with a central memory phenotype (probably related to IL-7 in the hPL preparation).

Example 3: Autologous glioblastoma dendritic cell vaccine

Dendritic cell (DC) vaccines have been proven safe and efficacious in treating cancer.[63] As an alternative to FBS, Švajger et al.[64] introduced hPL for the isolation and propagation of fully functional monocyte-derived DCs. Date et al.[65] documented that hPL even potentiates the properties of DCs matured with interferon-gamma. A recent study described the use of hPL in establishing a cell bank of clinical-grade human glioblastoma cell lines to serve as antigen sources for DC vaccines.[66] hPL accelerated the growth of glioblastoma cells, which expressed more stem cell markers and maintained better genetic stability, as compared to cells expanded in neural stem cell medium or FBS-supplemented medium. Monocyte-derived DC cultures were optimised, comparing human serum-supplemented media with commercial serum-free media; this resulted in a proprietary media formulation.

Discussion

Calls for better scientific reproducibility, an increasing awareness of the effects of serum/serum batches on cell behaviour and data variation, as well as the increase in cell-based therapies, have all led to greater consideration of how best to culture cells *in vitro*. FBS in particular is under scrutiny, and alternatives (e.g. serum-free, xeno-free, animal component-free, protein-free media and chemically defined media) are needed.[16] The use of human blood components have been suggested, especially for the commercial manufacturing of cell-based therapies. However, it should be noted that human supplements share many of the disadvantages of FBS: hPL and human serum are still ill-defined, suffer from batch-to-batch variation, and pose a risk of pathogen transmission or an immune system reaction. However, they probably represent an interim solution towards 'Good Cell Culture Practices'. The development of fully chemically-defined media appears to have been particularly successful in recent years.[67] However, the composition of any such medium needs to be carefully controlled to ensure that it really is entirely animal-component/xeno-free, and does not contain other factors such as hPL.[16]

By providing three different scenarios and a range of examples in each, we hope to encourage researchers to transition to animal component-free or, even better, to chemically defined media. Numerous examples of suitable media for various cell lines are available. There is always the plea of 'never change a winning team', but researchers need to become aware that poor reproducibility is doing science no good. And how much does short-term 'winning' benefit a team that might not run at all at the next round?

Outlook

The increasing awareness of not only researchers, industry and publishers, but also governmental bodies — in conjunction with, importantly, funding in the field of the Three Rs — give a strong impetus towards animal component-free cell culture. Thus, we recommend, especially when establishing new cell culture protocols, that some time is spent in the library evaluating existing and tried replacements to animal-derived cell culture components. As the US chemist Frank Henry Westheimer was famously quoted to have said: "A couple of months in the laboratory can frequently save a couple of hours in the library".

To avoid the use of FBS from the very beginning of a research project, existing databases are a good starting point. The Fetal Calf Serum-Free Database (https://fcs-free.org/) provides an overview of FCS-free media for a variety of cell types. Up to September 2021, more than 1500 different cell culture media for more than 250 cell types had already been listed. Given that there are alternatives to FBS, its unquestioned and continuous use is no longer justified. Rather, it should be justified to demand high precision and reliability in cell culture research, as is the custom in most other fields of the natural sciences.

Furthermore, even though there might (yet) be cost differences between cell culture supplements, choosing a lower priced product that is also ethically questionable,

unregulated and undefined can have far a greater negative financial effect on research work due to the need for optimisation, frequent experimental repetition, latent scientific inaccuracies and potential lack of reproducibility.[68] Let us be honest here: every researcher, scientific journal and, in the end, patient who will need the pharmaceutical for treatment, all want reproducible results. Therefore, if science wants to step out of its current reproducibility crisis, it can't go on employing irreproducible media formulations.

The ideal situation would be to establish and verify a serum-free approach from the outset. In the meantime, it would currently represent an immense step forward when peer-reviewers demand to verify experimental results in a xeno-free or chemically defined environment. At the very least, scientific journals should demand justification for the use of animal-derived products (under the ethical statements, as an acknowledgement of awareness) and ask how researchers are thinking forwards towards a replacement.

Progress is not made by travelling the well-worn paths, but instead by seeking out and finding new, better and sustainable means of improvement. We hope that the examples given here will not only serve to highlight the possibilities, but also inspire new developments and innovations for the benefit of researchers, industry and patients alike.

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References

1. Yao T and Asayama Y. Animal-cell culture media: History, characteristics, and current issues. *Reprod Med Biol* 2017; 16: 99–117.
2. Baker M. 1,500 scientists lift the lid on reproducibility. *Nature* 2016; 533: 452–454.
3. Baker M. Reproducibility: Respect your cells! *Nature* 2016; 537: 433–435.
4. Jochems CEA, van der Valk JBF, Stafleu FR, et al. The use of fetal bovine serum: Ethical or scientific problem? *Altern Lab Anim* 2002; 30: 219–227.
5. van der Valk J, Mellor D, Brands R, et al. The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicol In Vitro* 2004; 18: 1–12.
6. Gstraunthaler G, Lindl T and van der Valk J. A plea to reduce or replace fetal bovine serum in cell culture media. *Cytotechnology* 2013; 65: 791–793.
7. Gstraunthaler G, Lindl T and van der Valk J. A severe case of fraudulent blending of fetal bovine serum strengthens the case for serum-free cell and tissue culture applications. *Altern Lab Anim* 2014; 42: 207–209.
8. van der Valk J, Bieback K, Buta C, et al. Fetal Bovine Serum (FBS): Past — Present — Future. *ALTEX* 2018; 35: 99–118.
9. Even MS, Sandusky CB and Barnard ND. Serum-free hybridoma culture: Ethical, scientific and safety considerations. *Trends Biotechnol* 2006; 24: 105–108.
10. Marcus-Sekura C, Richardson JC, Harston RK, et al. Evaluation of the human host range of bovine and porcine viruses that may contaminate bovine serum and porcine trypsin used in the manufacture of biological products. *Biologicals* 2011; 39: 359–369.
11. Bieback K, Fernandez-Muñoz B, Pati S, et al. Gaps in the knowledge of human platelet lysate as a cell culture supplement for cell therapy: A joint publication from the AABB and the International Society for Cell & Gene Therapy. *Cytotherapy* 2019; 21: 911–924.
12. Versteegen RJ, Murray J and Doelger S. Animal welfare and ethics in the collection of fetal blood for the production of fetal bovine serum. *ALTEX* 2021; 38: 319–323.
13. Weber T, Wirths F, Brakebusch N, et al. Reply to comment 'Animal welfare and ethics in the collection of fetal blood for the production of fetal bovine serum'. *ALTEX* 2021; 38: 324–326.
14. Kinzebach S and Bieback K. Expansion of mesenchymal stem/stromal cells under xenogenic-free culture conditions. *Adv Biochem Eng Biotechnol* 2013; 129: 33–57.
15. Cassotta M, Pistollato F, Bartnicka JJ, et al. A worldwide survey on the use of animal-derived materials and reagents in scientific experimentation. *Eng Life Sci* 2022; 1–20.
16. Karnieli O, Friedner OM, Allickson JG, et al. A consensus introduction to serum replacements and serum-free media for cellular therapies. *Cytotherapy* 2017; 19: 155–169.

17. van der Valk J, Brunner D, De Smet K, et al. Optimization of chemically defined cell culture media — Replacing fetal bovine serum in mammalian *in vitro* methods. *Toxicol In Vitro* 2010; 24: 1053–1063.
18. Bieback K. Platelet lysate as replacement for fetal bovine serum in mesenchymal stromal cell cultures. *Transfus Med Hemother* 2013; 40: 326–335.
19. Strunk D, Lozano M, Marks DC, et al. International Forum on GMP-grade human platelet lysate for cell propagation: Summary. *Vox Sang* 2018; 113: 80–87.
20. Moreira F, Mizukami A, de Souza LEB, et al. Successful use of human AB serum to support the expansion of adipose tissue-derived mesenchymal stem/stromal cell in a microcarrier-based platform. *Front Bioeng Biotechnol* 2020; 8: 307.
21. Jacobs MN, Versteegen RJ, Treasure C, et al. Addressing potential ethical issues regarding the supply of human-derived products or reagents in *in vitro* OECD Test Guidelines. *ALTEX* 2019; 36: 163–176.
22. Schallmoser K, Henschler R, Gabriel C, et al. Production and quality requirements of human platelet lysate: A position statement from the working party on cellular therapies of the international society of blood transfusion. *Trends Biotechnol* 2020; 38: 13–23.
23. Mentari D, Pebrina R and Nurpratami D. Utilization of expired platelet concentrate for production of human platelet lysate as a medium for T47D cell propagation. *Cell Biomed Sci* 2022; 6: 96–103.
24. Burgener A and Butler M. Medium development. In: Ozturk SS, Hu W-S (eds) *Cell culture technology for pharmaceutical and cell-based therapies*. Boca Raton: Taylor & Francis, 2006, pp. 41–79.
25. Lee GM, Kim EJ, Kim NS, et al. Development of a serum-free medium for the production of erythropoietin by suspension culture of recombinant Chinese hamster ovary cells using a statistical design. *J Biotechnol* 1999; 69: 85–93.
26. Knöspel F, Schindler RK, Lübberstedt M, et al. Optimization of a serum-free culture medium for mouse embryonic stem cells using design of experiments (DoE) methodology. *Cytotechnology* 2010; 62: 557–571.
27. Zhao A, Chen F, Ning C, et al. Use of real-time cellular analysis and Plackett-Burman design to develop the serum-free media for PC-3 prostate cancer cells. *PLoS One* 2017; 12: 1–16.
28. Lerman MJ, Lembong J, Muramoto S, et al. The evolution of polystyrene as a cell culture material. *Tissue Eng Part B Rev* 2018; 24: 359–372.
29. Lepe-Zuniga JL, Zigler Jr JS and Gery I. Toxicity of light-exposed Hepes media. *J Immunol Methods* 1987; 103: 145.
30. Curtis ASG, Forrester JV, McInnes C, et al. Adhesion of cells to polystyrene surfaces. *J Cell Biol* 1983; 97: 1500–1506.
31. Yao T and Asayama Y. Animal-cell culture media: History, characteristics, and current issues. *Reprod Med Biol* 2017; 16: 99–117.
32. Pamies D, Leist M, Coecke S, et al. Guidance document on Good Cell and Tissue Culture Practice 2.0 (GCCP 2.0). *ALTEX* 2022; 39: 30–70.
33. Marigliani B, Balottin LBL and Augusto E de FP. Adaptation of mammalian cells to chemically defined media. *Curr Protoc Toxicol* 2019; 82: e88.
34. Lindl T and Steubing R. *Atlas of living cell cultures*. Weimheim: Wiley-VCH, 2013, 507 pp.
35. Earle WR, Schilling EL, Stark TH, et al. Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells. *JNCI J Natl Cancer Inst* 1943; 4: 165–212.
36. Sanford KK, Earle WR and Likely GD. The growth *in vitro* of single isolated tissue cells. *J Natl Cancer Inst* 1948; 9: 229–246.
37. Kiesslich S and Kamen AA. Vero cell upstream bioprocess development for the production of viral vectors and vaccines. *Biotechnol Adv* 2020; 44: 107608.
38. Rourou S, Ben Zakkour M and Kallel H. Adaptation of Vero cells to suspension growth for rabies virus production in different serum free media. *Vaccine* 2019; 37: 6987–6995.
39. Fusi M and Dotti S. Adaptation of the HEp-2 cell line to totally animal-free culture systems and real-time analysis of cell growth. *Biotechniques* 2021; 70: 319–326.
40. Brischwein M and Wiest J. Microphysiometry. In: Wegener J (ed) *Label-free monitoring of cells in vitro*. Cham: Springer International Publishing, 2019, pp. 163–188.
41. Klenk C and Wiest J. Cellasys #8: A microphysiometric assay to asses enhanced cell culture media. In: *ALTEX Proceedings 9(1).* Switzerland: ALTEX Edition, 2021, p. 207.
42. Schmidt C and Wiest J. Three-stage approach for evaluation of a chemically defined cell culture medium for the Caco-2 cell line: Short-term effects, differentiation potential and long-term cultivation. In: *ALTEX Proceedings 9(1).* Switzerland: ALTEX Edition, 2021, p. 215.
43. Weber T, Wagner K and Wiest J. Chemically defined cell culture media — a contribution to address the reproducibility crisis in biomedical sciences. In: *ALTEX Proceedings 9(1).* Switzerland: ALTEX Edition, 2021, p. 262.
44. Schaffner AE, Barker JL, Stenger DA, et al. Investigation of the factors necessary for growth of hippocampal neurons in a defined system. *J Neurosci Methods* 1995; 62: 111–119.
45. Guo X, Gonzalez M, Stancescu M, et al. Neuromuscular junction formation between human stem cell-derived motoneurons and human skeletal muscle in a defined system. *Biomaterials* 2011; 32: 9602–9611.
46. Smith A, Long C, Mcaleer C, et al. Chapter 7: 'Body-on-a-chip' technology and supporting microfluidics. In: *RSC Drug Discovery Series, Human-based systems for translational research*. London: Royal Society of Chemistry, 2015, pp. 132–161.
47. Slaughter VL, Rumsey JW, Boone R, et al. Validation of an adipose-liver human-on-a-chip model of NAFLD for

preclinical therapeutic efficacy evaluation. *Sci Rep* 2021; 11: 13159.
48. Palikuqi B, Nguyen D-HT, Li G, et al. Adaptable haemodynamic endothelial cells for organogenesis and tumorigenesis. *Nature* 2020; 585: 426–432.
49. Sriram G, Handral HK, Gan SU, et al. Fabrication of vascularized tissue constructs under chemically defined culture conditions. *Biofabrication* 2020; 12: 45015.
50. Galipeau J and Sensébé L. Mesenchymal stromal cells: Clinical challenges and therapeutic opportunities. *Cell Stem Cell* 2018; 22: 824–833.
51. Wilson AJ, Rand E, Webster AJ, et al. Characterisation of mesenchymal stromal cells in clinical trial reports: Analysis of published descriptors. *Stem Cell Res Ther* 2021; 12: 360.
52. Mendicino M, Bailey AM, Wonnacott K, et al. MSC-based product characterization for clinical trials: An FDA perspective. *Cell Stem Cell* 2014; 14: 141–145.
53. Mannello F and Tonti GA. Concise review: No breakthroughs for human mesenchymal and embryonic stem cell culture: Conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or had hoc formula? All that glitters is not gold! *Stem Cells* 2007; 25: 1603–1609.
54. Burnouf T, Strunk D, Koh MBC, et al. Human platelet lysate: Replacing fetal bovine serum as a gold standard for human cell propagation? *Biomaterials* 2016; 76: 371–387.
55. Kocaoemer A, Kern S, Klüter H, et al. Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. *Stem Cells* 2007; 25: 1270–1278.
56. Bieback K, Hecker A, Schlechter T, et al. Replicative aging and differentiation potential of human adipose tissue-derived mesenchymal stromal cells expanded in pooled human or fetal bovine serum. *Cytotherapy* 2012; 14: 570–583.
57. Bieback K, Ha VA-T, Hecker A, et al. Altered gene expression in human adipose stem cells cultured with fetal bovine serum compared to human supplements. *Tissue Eng Part A* 2010; 16: 3467–3484.
58. Dreher L, Elvers-Hornung S, Brinkmann I, et al. Cultivation in human serum reduces adipose tissue-derived mesenchymal stromal cell adhesion to laminin and endothelium and reduces capillary entrapment. *Stem Cells Dev* 2013; 22: 791–803.
59. Kinzebach S, Dietz L, Klüter H, et al. Functional and differential proteomic analyses to identify platelet derived factors affecting *ex vivo* expansion of mesenchymal stromal cells. *BMC Cell Biol* 2013; 14: 48.
60. Kwon D, Kim J-S, Cha B-H, et al. The effect of fetal bovine serum (FBS) on efficacy of cellular reprogramming for induced pluripotent stem cell (iPSC) generation. *Cell Transplant* 2016; 25: 1025–1042.
61. Torres Chavez A, McKenna MK, Canestrari E, et al. Expanding CAR T cells in human platelet lysate renders T cells with *in vivo* longevity. *J Immunother Cancer* 2019; 7: 330.
62. Canestrari E, Steidinger HR, McSwain B, et al. Human platelet lysate media supplement supports lentiviral transduction and expansion of human T lymphocytes while maintaining memory phenotype. *J Immunol Res* 2019; 2019: 3616120.
63. Perez CR and De Palma M. Engineering dendritic cell vaccines to improve cancer immunotherapy. *Nat Commun* 2019; 10: 5408.
64. Švajger U. Human platelet lysate is a successful alternative serum supplement for propagation of monocyte-derived dendritic cells. *Cytotherapy* 2017; 19: 486–499.
65. Date I, Koya T, Sakamoto T, et al. Interferon-α-induced dendritic cells generated with human platelet lysate exhibit elevated antigen presenting ability to cytotoxic T lymphocytes. *Vaccines* 2020; 9: 10.
66. Parney IF, Gustafson MP, Solseth M, et al. Novel strategy for manufacturing autologous dendritic cell/allogeneic tumor lysate vaccines for glioblastoma. *Neurooncol Adv* 2020; 2: vdaa105.
67. Bhat S, Viswanathan P, Chandanala S, et al. Expansion and characterization of bone marrow derived human mesenchymal stromal cells in serum-free conditions. *Sci Rep* 2021; 11: 3403.
68. Weber T and Wagner K. Replacing fetal bovine serum (FBS) in research and testing. *ALTEX* 2021; 38: 163–164.