Comment

Fetal Bovine Serum (FBS) — A Pain in the Dish?

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The use of Fetal Bovine Serum in replacement alternative methods is associated with serious animal welfare concerns, as well as worrying reproducibility issues

Fetal Bovine Serum (FBS) is still the most commonly used cell and tissue culture media supplement. Since it contains essential components for the proliferation and maintenance of most cell types, FBS can be regarded as the universal growth supplement to basal culture media. Many scientists who are involved in *in vitro* research, supplement their media with FBS, without realising that FBS is actually serum obtained from a *living* unborn calf. Even fewer know that the collection of blood from the unborn calf can involve suffering.[1,2] Drawing blood from a living fetus, mostly through a heart puncture, can cause suffering, in particular when it takes place in the last third of its fetal development and without any precautions taken to minimise suffering.[2] Examples of such precautions include: a) ensuring that fetal blood collection starts at least five minutes after a complete neck incision in the dam has been completed, and the electroencephalographic or electrocorticographic (EEG/ECoG) patterns are flat and remain flat; and b) not allowing the fetus to breathe after removal from the dam; or c) stunning the fetus with a captive bolt firearm or a blow to the head with a suitable blunt object.[2] Although these measures can in theory be taken to minimise suffering, there is no indication that they are actually applied, let alone subject to inspection by the authorities. So, it can be assumed that FBS production involves animal suffering. Therefore, since a wide range of *in vitro* models have been (and are currently being) developed to replace or reduce animal experiments and animal suffering, the use of FBS in these methods seems at odds with the Three Rs principles.[3]

In addition, particularly when blood collection takes place on EU territory, the question is whether it ought to be subject to a formal project review: i.e. the calves are killed for scientific purposes in their last third of embryonic development, so should the procedure, which involves equal or more suffering than the injection of a needle, not be regarded as an animal experiment?[4,5]

A way to get around this ethical problem is to supplement media with serum-like compounds that do not involve animal suffering. One of these is human platelet lysate (hPL). Although the use of hPL is becoming increasingly popular as a universal supplement, it unfortunately does not fully address the scientific issues related to the use of FBS.[5]

Reproducibility issues

Although serum from a fetal calf seems to contain all the necessary components to suit most cells maintained outside a body in artificial surroundings, it contains several factors that might detrimentally influence the outcome of experiments. FBS is a complex biological product of unknown composition, with seasonal and geographical fluctuations, and it therefore differs between different lots, even from the same manufacturer. This is supported by the observation that cells grown and maintained in different batches of serum, thus with different compositions, give different results under the same culture conditions. The search for a new batch of FBS, in which cell growth, behaviour and other properties are similar as compared to the old batch, is costly and time-consuming. So, reproducibility is at stake, both within a given laboratory, and certainly between laboratories. Not only is this scientifically questionable, but when a method involving FBS as a medium supplement is expected to be applied in a regulatory context, it is actually almost useless. It would be nearly impossible for all testing laboratories to use the same batch of FBS for their experiments, as each batch of FBS is limited by its finite volume. Furthermore, it would be almost impossible to find a batch that gives similar results to the reference medium, since many different batches of serum would have to be painstakingly tested in order to potentially achieve this. Thus, the use of FBS implicitly introduces issues of reproducibility, both within one laboratory over time, and also between laboratories at all times.[6,7]

Other issues associated with the use of FBS

In addition to its use as a supplement in cell and tissue culture media for research, FBS is also commonly used in applications such as processed meat production, *in vitro* fertilisation, cell therapy, cell banking, and for the production of recombinant products and therapeutics (especially vaccines). The resulting year-on-year increase in the use of FBS has already led to a situation in which the demand exceeds the supply.[8,9] This is further illustrated by a 300% increase in the price of FBS.[9] The shortage in FBS supply is a serious concern for its future use.

Another worrying issue, besides variability in its composition and supply issues, is the possible contamination with viruses, bacteria, mycoplasma and other adventitious factors. A recent study that tested 20 commercial FBS batches from 12 suppliers found that only one batch was virus-free and others were contaminated with between one and 11 viruses.[10] The BSE outbreak, which started in 1985 and mostly affected the UK and France, significantly contributed to the experimental reproducibility crisis, due to its effect on the supply and possible contamination of FBS. The outbreak also increased human health risks, as a result of the use of FBS in the production of, for instance, vaccines.[11] Furthermore, since FBS might induce subtle and unexpected modifications of stem cells, it should be used with caution in cell therapeutic use.[6]

Solutions

The preferred medium for use in cell culture procedures is animal component-free and chemically-defined. Although chemically-defined media are available for several cell types, these are selective and generally only appropriate for use with one specific cell type. The development of media for other cell types is possible, but can prove to be time consuming.[12]

In the meantime, to overcome ethical, legal and supply issues, sera substitutes should be considered. hPL is becoming popular and has been used in several situations.[12,13] hPLs are obtained from donated human thrombocyte units that are past their expiry date. Therefore, hPL is clinically tested, xeno-free and it contains those growth factors essential for cell growth and proliferation *in vitro*. So far, hPL has been shown to support the growth and proliferation of all of the animal and human cell lines tested, with both anchorage-dependent and suspension growth characteristics. Furthermore, in terms of rates of proliferation, hPL was superior to FBS, which currently is the only benchmark available. Thus, hPL can be seen as a good universal alternative for dealing with the welfare aspects of FCS use. It could also be an alternative in large scale production facilities, when no other alternatives exist.

The use of chemically-defined media has been proposed as the ultimate solution to the problems described above.[14,15] Such media have several advantages: their composition is defined and controlled, leading to low qualitative and quantitative variability, and they contain no adverse factors (e.g. bacteria and viruses). As yet, no universal chemically-defined medium has been developed — instead, the medium is developed for a specific cell type. This specificity could be a major advantage, particularly for primary cell cultures, where there is a risk that unwanted cells (mostly fibroblasts) can outcompete and overgrow the required cells. Tailored chemically-defined media also offer the possibility of selecting for specific cell types. The development of such media and possible adaptation strategies for particular cell types have been described in a workshop report, published in 2010.[12]

Serum-free media have been developed for several cell types and cell lines. Several of these are described in the literature, and others are commercially available. Although they are often indicated as chemically-defined, the formulations of commercially-available media are, unfortunately, not always released. To facilitate the identification of serum-free media for specific cell types, an FCS-free database (https://fcs-free.org/) has been launched. This database recently became available online, and includes interesting features, such as a 'Comment' function for each record in the database, through which improvements to existing media can be discussed. Together, we should be able to eradicate the use of FBS in cell and tissue cultures, thereby contributing not only to animal welfare, but also to better scientific methods and more-reproducible *in vitro* results.[7]

Recommendations

Many new methods involving a range of cell and tissue culture techniques are currently in development, or will be developed in the near future. When these new methods are being developed, or existing methods are being validated, the use of serum-free media should be considered at all times. In 2008, EURL ECVAM released a statement urging that, whenever possible, non-animal substitutes for FBS should be used, especially when new *in vitro* culture test methods are being developed.[16] In addition, funding bodies should apply the FBS-free principle as a funding requirement, and they might also consider the inclusion of extra funding for the development of a serum-free medium when one does not already exist for the cell type proposed for use in the *in vitro* method. Regulatory bodies should require that the media used in new methods are, at the very least, FBS-free, but preferably that they are chemically-defined and animal component-free. Existing methods based on the use of FBS supplements should be adapted to work under serum-free conditions.

The use of FBS is not the only worrying factor affecting the validity of scientific results obtained from *in vitro* cell and tissue cultures. The misidentification of cell lines is also a serious concern.[16,17] In his 'Food for thought…' series in *ALTEX*, Thomas Hartung discussed many other issues related to *in vitro* research that should be considered and could be improved upon.[19]

A comprehensive guide to good *in vitro* method practices (GIVIMP) will shortly be published by the Organisation for Economic Co-operation and Development (OECD).[20] The aim of this guidance document will be to improve "the reliability and robustness of *in vitro* methods, reducing the uncertainties of *in vitro*-based predictions and therefore increasing the acceptance of the *in vitro* estimated safety measures by regulatory agencies".[21] The guide will provide a comprehensive overview of most issues related to *in vitro* research.

Currently, we are seeing a continuous increase in the application of *in vitro* methods, and a consequent rise in the use of, and demand for, FBS. Therefore, the ethical, scientific (i.e. reproducibility and safety) and availability issues related to FBS use should be taken seriously, and the development and use of serum-free (preferably chemically-defined) media should be considered at all times. This important issue is further extensively discussed in a comprehensive consensus report.[5]

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References

1 Jochems, C.E., van der Valk, J.B., Stafleu, F.R. & Baumans, V. (2002). The use of fetal bovine serum: Ethical or scientific problem? *ATLA* **30**, 219–227.

2 van der Valk, J., Mellor, D., Brands, R., Fischer, R., Gruber, F., Gstraunthaler, G., Hellebrekers, L., Hyllner, J., Jonker, F.H., Prieto, P., Thalen, M. & Baumans, V. (2004). The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicology in Vitro* **18**, 1–12.

3 Russell, W.M.S. & Burch, R.L. (1959). *The Principles of Humane Experimental Technique*, 238pp. London, UK: Methuen.

4 Anon. (2010). *Directive 2010/63/EU* of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union* **L276**, 20.10.2010, 33–79.

5 van der Valk, J., Bieback, K., Buta, C., Cochrane, B., Dirks, W.G., Fu, J., Hickman, J.J., Hohensee, C., Kolar, R., Liebsch, M., Pistollato, F., Schulz, M., Thieme, D., Weber, T., Wiest, J., Winkler, S. & Gstraunthaler, G. (2018). Fetal bovine serum (FBS): Past–Present–Future. *ALTEX*, doi:10.14573/altex.1705101. [E-pub ahead of print.]

6 Mannello, F. & Tonti, G.A. (2007). 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 *ad hoc* formula? All that glitters is not gold! *Stem Cells* **25**, 1603–1609.

7 Baker, M. (2016). Reproducibility: Respect your cells! *Nature, London* **537**, 433–435.

8 Brindley, D.A., Davie, N.L., Culme-Seymour, E.J., Mason, C., Smith, D.W. & Rowley, J.A. (2012). Peak serum: Implications of serum supply for cell therapy manufacturing. *Regenerative Medicine* **7**, 7–13.

9 Fang, C-Y., Wu, C-C., Fang, C-L., Chen, W-Y. & Chen, C-L. (2017). Long-term growth comparison studies of FBS and FBS alternatives in six head and neck cell lines. *PLoS One* **12**, e0178960.

10 Toohey-Kurth, K., Sibley, S.D. & Goldberg, T.L. (2017). Metagenomic assessment of adventitious viruses in commercial bovine sera. *Biologicals* **47**, 64–68.

11 US FDA (2013). *Bovine derived materials used in vaccine manufacturing questions and answers*. Silver Spring, MD, USA: US Food and Drug Administration. Available at: https://www.fda.gov/BiologicsBloodVaccines/Vaccines/QuestionsaboutVaccines/ucm143521.htm (Accessed 31.10.17).

12 van der Valk, J., Brunner, D., De Smet, K., Fex Svenningsen, A., Honegger, P., Knudsen, L.E., Lindl, T., Noraberg, J., Price, A., Scarino, M.L. & Gstraunthaler, G. (2010). Optimization of chemically defined cell culture media — replacing fetal bovine serum in mammalian *in vitro* methods. *Toxicology in Vitro* **24**, 1053–1063.

13 Gstraunthaler, G., Rauch, C., Feifel, E. & Lindl, T. (2015). Preparation of platelet lysates for mesenchymal stem cell culture media. *Journal of Stem Cells Research, Reviews & Reports* **2**, 1021.

14 Barnes, D. & Sato, G. (1980). Methods for growth of cultured cells in serum-free medium. *Analytical Biochemistry* **102**, 255–270.

15 Barnes, D. & Sato, G. (1980). Serum-free cell culture: A unifying approach. *Cell* **22**, 649–655.

16 ESAC (2008). *ESAC statement on the use of FCS and other animal-derived supplements*. Ispra, Italy: European Centre for the Validation of Alternative Methods. Available at: https://eurl-ecvam.jrc.ec.europa.

eu/about-ecvam/archive-publications/publication/ESAC28_statement_FCS_20080508.pdf (Accessed 01.11.17).

[17] Castro, F., Dirks, W.G., Fähnrich, S., Hotz-Wagenblatt, A., Pawlita, M. & Schmitt, M. (2013). High-throughput SNP-based authentication of human cell lines. *International Journal of Cancer* **132**, 308–314.

[18] Geraghty, R.J., Capes-Davis, A., Davis, J.M., Downward, J., Freshney, R.I., Knezevic, I., Lovell-Badge, R., Masters, J.R., Meredith, J., Stacey, G.N., Thraves, P. & Vias, M.; Cancer Research UK (2014). Guidelines for the use of cell lines in biomedical research. *British Journal of Cancer* **111**, 1021–1046.

[19] Hartung, T. (2007). Food for thought... on cell culture. *ALTEX* **24**, 143–152.

[20] OECD (undated). *Draft Guidance Document on Good* In Vitro *Method Practices (GIVIMP) for the Development and Implementation of* In Vitro *Methods for Regulatory Use in Human Safety Assessment*, 112pp. Paris, France: Organisation for Economic Co-operation and Development. Available at: http://www.oecd.org/env/ehs/testing/OECD_Draft_GIVIMP_in_Human_Safety_Assessment.pdf (Accessed 01.11.17).

[21] Anon. (2017). *EUROTOX 2017, ESTIV GIVIMP/GCCP Session Abstract.* Available at: http://www.eurotox2017.com/tuesday/asss-1/ (Accessed 14.11.17).