

The Overt and Hidden Use of Animal-derived Products in Alternative Methods for Skin Sensitisation: A Systematic Review

Alternatives to Laboratory Animals
1–22

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Abstract

In vitro methods that can replace animal testing in the identification of skin sensitisers are now a reality. However, as cell culture and related techniques usually rely on animal-derived products, these methods may be failing to address the complete replacement of animals in safety assessment. The objective of this study was to identify the animal-derived products that are used as part of *in vitro* methods for skin sensitisation testing. Thus, a systematic review of 156 articles featuring 83 different *in vitro* methods was carried out and, from this review, the use of several animal-derived products from different species was identified, with the use of fetal bovine serum being cited in most of the methods (78%). The use of sera from other animals, monoclonal antibodies and animal proteins were also variously mentioned. While non-animal alternatives are available and methods free of animal-derived products are emerging, most of the current methods reported used at least one animal-derived product, which raises ethical and technical concerns. Therefore, to deliver technically and ethically better *in vitro* methods for the safety assessment of chemicals, more effort should be made to replace products of animal origin in existing methods and to avoid their use in the development of new method protocols.

Keywords

allergic contact dermatitis, alternatives to animal testing, bioethics, biological products, dermatitis, Three Rs

Introduction

Topically applied substances can give rise to allergic reactions.¹ Contact allergy is a complex disease, with its increasing prevalence leading to high costs for healthcare systems.² In order to evaluate the safety of cosmetics, it is mandatory to identify the sensitisation potential of substances, which was historically carried out by using animals, for example, the Guinea Pig Maximisation Test³ and the Local Lymph Node Assay^{4–6}; the latter has been preferred by regulatory authorities for several years.⁷ However, *in vivo* tests are expensive, time-consuming, ethically controversial and provide potentially questionable results in terms of transferability to humans.⁸ Thus, there is a worldwide effort to perform safety assessments without the use of animals, for example, the European Union implemented a staged ban on the sale of cosmetics tested on animals (starting with a ban on finished products in 2004,

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then ingredients in 2009 and the full ban in 2013),⁹ and similar legislation is now a reality in many other countries.¹⁰

Advances in the understanding of cellular and molecular mechanisms involved in the skin sensitisation process, together with new technological platforms, have provided opportunities to create new methods and strategies. Particularly in the field of skin sensitisation prediction, considerable progress has been made in the development of different approaches (including *in vitro*, *in chemico* and *in silico* methods).^{11–13} Skin sensitisation is a complex immunological process elicited by the covalent binding of substances to skin proteins. In the induction phase, the adverse outcome pathway (AOP) for skin sensitisation includes the activation of keratinocytes, the processing of the hapten–protein complex by skin dendritic cells (DCs) and the presentation of parts of the processed complex to naïve T-cells, inducing differentiation and proliferation of specific memory T-cells.¹⁴

Due to the complexity of the process, a combination of methods is needed to replace animal use in the identification of skin sensitisers.^{13,15} These methods must apply the mechanistic understanding of the immunological process,¹⁶ incorporating the assessment of the key steps of the AOP. In a regulatory decision-making process, it is expected that integrated approaches to testing and assessment can efficiently combine results from current *in silico*, *in chemico* and *in vitro* methods with other relevant complementary information (e.g. physico-chemical properties), to provide reliable information.^{13,17,18} As the non-animal safety assessment of skin sensitisation combines several *in vitro* methods, and as these methods typically use animal-derived products, we may be failing to address the complete replacement of animals in skin sensitisation safety assessment.

One of the most commonly used animal-derived products in these methods is fetal bovine serum (FBS), which is also referred to as fetal calf serum (FCS). (It should be noted, however, that neither FBS nor FCS are the same as bovine calf serum.) FBS is added to cell culture medium as a supplement, mainly as a source of hormones and growth factors. To harvest FBS, bovine fetuses are taken from pregnant cows sent for slaughter, and the blood of the fetus is collected by cardiac puncture without anaesthesia. As the animal is expected to be alive during this procedure, the potential for suffering should be strongly considered.^{19,20} In addition, the use of FBS has other associated disadvantages, such as the risk of contamination, batch-to-batch variation and the introduction of many unknown variables.²¹ Its use in alternative *in vitro* methods has been increasingly criticised, not only for ethical but also for technical and scientific reasons. While the use of FBS was seen as crucial in the past, a reduction in its use, or preferably its replacement altogether, is now looking increasingly feasible.²²

Most *in vitro* methods for chemical safety assessment involve the exposure of human or animal tissues or cells (of primary origin or cell lines) to the test chemical, in order to determine the extent of its toxic effects. In skin sensitisation testing, the aim is to distinguish sensitising from non-sensitising chemicals. This aim can be achieved in a range of different ways, depending on the read-out parameter. Immunological techniques are commonly used to obtain these read-outs,²³ and these techniques tend to use antibodies that have historically been produced by using animals and several other animal-derived products.

If the *in vitro* methods that are being used to replace the *in vivo* studies employ animal-derived products, then we are not completely replacing animals in chemical safety assessment — but this is necessary in order to have truly cruelty-free cosmetics. Thus, there is a need for detailed information on what these products are, whether there are any associated technical and ethical issues, and whether animal-free replacements are indeed available. Therefore, the main objective of this systematic review was to identify the types of animal-derived products that are currently being used in existing *in vitro* methods that aim to replace the use of animals for the prediction of skin sensitisation.

It was not our objective to compile an extensive list of the existing *in vitro* methods for skin sensitisation, or to compare them, as this has already been done by various groups.^{11,12,24–27} Our aim was to identify the various animal-derived products that were being used in the methods, which turned out to be quite a challenging task, as not all of the animal-derived products used were clearly mentioned in the articles analysed, or the use of an animal-derived product was often completely omitted from the method description. Furthermore, it was the case that some types of product can often be derived from animals, but not exclusively so.

Methods

Search strategy

Methods aimed at reducing and/or refining animal use in testing were not included in this review. However, methods that used animals as a source of cells were included, even though they are not full replacement methods.

We searched MEDLINE[®] through PubMed, EMBASE, the Latin American and Caribbean Health Science Literature (LILACS), and Cochrane Library databases, as well as the Open Thesis and the Organisation for Economic Co-operation and Development (OECD) repositories. The literature search was performed in July 2015 and updated in March 2019 by using various defined words and open terms related to alternatives to animal testing, *in vitro* methods and skin sensitisation. No language restrictions or other limitations were applied. The reference list from each of

the selected articles was searched manually, for additional relevant papers.

Article selection

Selection of the articles was conducted by two reviewers independently, and any disagreements were resolved by consensus. The articles retrieved were evaluated for eligibility in a two-step process. Firstly, the title and the abstract were assessed, and those articles that clearly did not fulfil the inclusion criteria were excluded. However, if the abstract was unavailable but the title was clearly relevant, or if a potential article was difficult to classify as eligible for other reasons, then eligibility was assessed by analysing the full text. Secondly, full texts were assessed, and the specific reasons for the exclusion of each article were recorded.

The inclusion criterion was specifically: ‘articles with comprehensive descriptions of alternative *in vitro* methods based on the use of mammalian cells, to identify potential and/or potency of skin sensitisation’. Basic science papers and those that did not propose an *in vitro* method for skin sensitisation testing were excluded. Systematic and non-systematic reviews, comparative studies and articles on strategies for integrating different alternative methods were included and analysed after data extraction from the articles comprising a specific *in vitro* method, and those methods that had not been previously identified were included (additional records).

Data extraction and analysis

The ‘Methods’ sections of the articles that fulfilled the inclusion criteria were carefully analysed and the following variables were extracted by two independent reviewers: experimental platform; technique; read-out parameter; animal-derived products that were clearly mentioned; potentially animal-derived products that were mentioned in the paper (when the products that were mentioned are usually animal-derived, but no specific information was provided regarding their source in that particular case); and probable animal-derived products (animal-derived products that might have been used, but were not mentioned in the paper).

Because different articles might describe the same technique or a single article might refer to more than one technique, articles featuring the same method (particularly with regard to cell type, technique and read-out parameter) were grouped. Methods were assigned to one of four categories: (i) assessment of keratinocyte activation; (ii) assessment of DC response; (iii) assessment of T-cell response; and (iv) methods based on reconstructed epidermis, skin models or explants (RSE). Only the main experimental platforms, techniques or read-out parameters — or those showing

promising results — were included in the analysis. Techniques used only for internal validation were not included.

Results

Articles and methods

A total of 850 articles were retrieved (Figure 1), comprising 739 found by searching the databases directly and 111 further papers identified from their reference lists. After excluding 83 duplicates, the titles and abstracts of 767 articles were assessed. A total of 560 articles clearly did not fulfil the inclusion criteria and thus were excluded. After reading the full text of the remaining papers, 51 were excluded: 21 either were not about *in vitro* methods, used non-mammalian cells, or were not about skin sensitisation testing; 26 were opinion pieces, state-of-the-art or other reports, with no comprehensive description of the methods; and the full text of the remaining four articles was unfortunately unavailable. A total of 156 articles met all of the inclusion criteria and these publications were analysed in more detail, revealing that a total of 83 different methods were featured. These methods were assessed and certain characteristics recorded, as shown in Tables 1 to 4, namely: the experimental platform; the read-out parameters; and specific techniques. The assessment then looked at the use of animal-derived products in detail, noting any that were:

- a. clearly mentioned as such in the article;
- b. mentioned and potentially animal-derived (i.e. the source was not specified in the article); and
- c. probably used (based on the type of technique described), but were not actually mentioned.

Of the 83 methods, eight assessed keratinocyte activation (Table 1), 50 were based on the assessment of DC response (Table 2), eight analysed T-cell response (Table 3) and 17 were based on RSE and related models (Table 4).

A variety of experimental platforms were identified, including human and non-human primary cells, cell lineages and skin explants. The read-out parameters included: activation of regulatory pathways; production of inflammatory cytokines; expression of surface markers; and cell migration. The specific techniques recorded were mainly histology; colorimetric and enzymatic assays; immunohistochemistry; reverse transcription polymerase chain reaction (RT-PCR); flow cytometry; and ³H-thymidine incorporation.

Some animal-derived products were clearly mentioned in the methods (e.g. FBS), but this was not the case for all. Some products, such as proteolytic enzymes (which are used to detach cells), might be of animal origin, but this uncertainty meant that we had to double-check on the manufacturer’s website, where this information was not always available. When there was no actual information available regarding the source, the products that are usually animal-

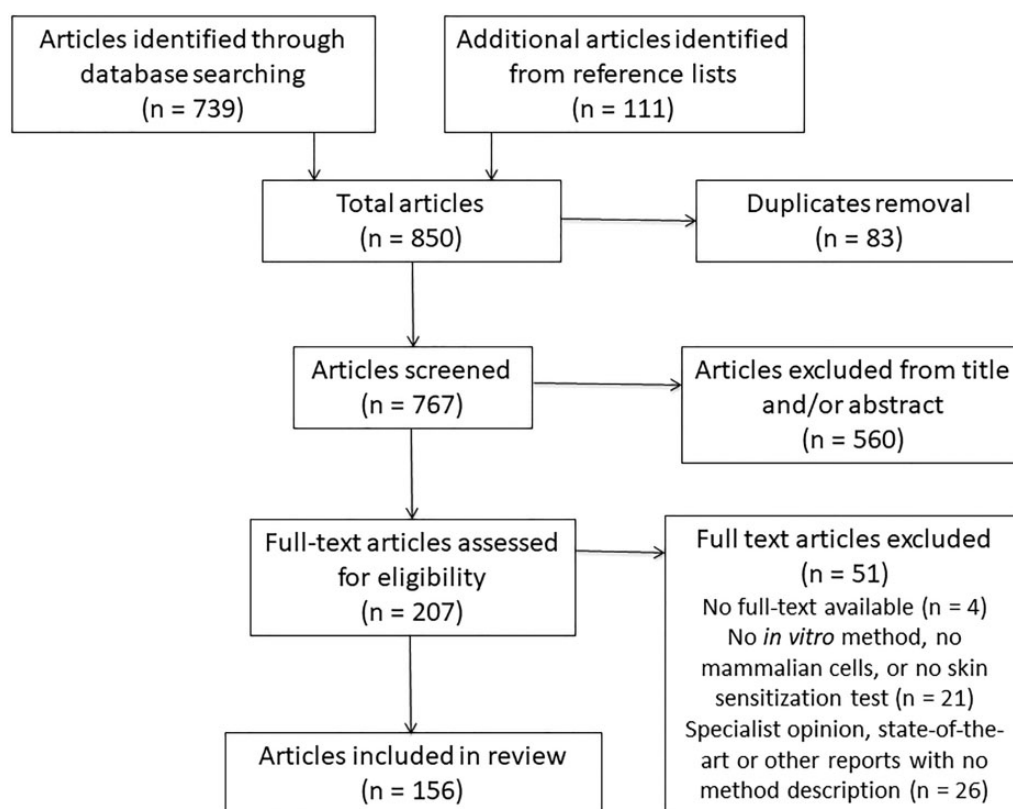


Figure 1. Systematic review flow chart of article selection.

derived (e.g. trypsin) were classified as ‘potentially animal-derived products’. In certain techniques, such as flow cytometry, the use of animal-derived products is so common that they are not routinely mentioned as such. This is the case for bovine serum albumin (BSA) and other animal-derived blocking agents. Therefore, such techniques were classified as involving the use of ‘probable animal-derived products’.

FBS was the most prevalent animal-derived product used. The use of animal sera, including FBS, and murine and rabbit sera, was clearly mentioned in all of the analysed methods to assess keratinocyte activation (Table 1), and in the vast majority (94%; 47/50) of the methods that assessed DC response (Table 2). Among the methods for assessing T-cell response (Table 3), 75% (6/8) used animal sera, and some used human serum. A total of 35% (6/17) of the methods based on RSE and related models used animal sera, including FCS, FBS and mouse and goat sera (Table 4). Overall, 78% of the methods analysed in this systematic review used at least one type of animal serum.

BSA was clearly mentioned only in 25% (2/8) of the methods to assess keratinocyte activation, in 40% (20/50) of those assessing DC response, in 37% (3/8) of methods assessing T-cell response and in 12% (2/17) of methods employing RSE and related models.

Trypsin was clearly mentioned by 62% (5/8) of the methods used to assess keratinocyte activation, 16%

(8/50) of the methods used to assess DC response and 12% (1/8) of methods used to assess T-cell response. However, trypsin use was not mentioned in any method employing RSE and related models (Table 4). In most articles, trypsin was mentioned without any details of the manufacturer or source, hence it was considered to be a ‘potentially animal-derived product’. In the other articles, the use of trypsin was not directly mentioned, but it was probably used in the culture of adherent cells or for dissociating tissues, and so the method was classified as ‘animal-derived products probably used’.

With regard to methods based on primary cells and cell lines, 37% (3/8) of such methods employed to assess keratinocyte activation, 4% (2/50) of those used to assess DC response, 13% (1/8) of those used to assess T-cell response and 6% (1/17) of those based on RSE and related models, featured the use of animal (i.e. murine)-derived cells.

The use of monoclonal and/or polyclonal antibodies was identified in 37% (3/8) of the methods to assess keratinocyte activation, 92% (46/50) of methods to assess DC responses, 100% (8/8) of the methods to assess T-cell response and 76% (13/17) of the methods based on RE and related models.

Heparin was identified in the description of some methods that used human cord blood (which was collected in tubes containing sodium heparin). In some cases, it was

Table 1. Details of the products of animal origin likely to have been used in the eight analysed *in vitro* methods for assessing keratinocyte activation.

Platform	Read-out parameter	Technique	Animal-derived products ^a	Potentially animal-derived products ^b	Probable animal-derived products ^c	References
Murine epidermal keratinocytes	Expression of CD40	Flow cytometry	Skin from back and ears of Balb/c mice, FBS	Trypsin; antibodies	Animal sera in trypsin neutralising solution; animal-derived blocking agent	28
C3H mouse-derived keratinocytes cell line HEL30	IL-18 and/or IL-1 α production	PCR and/or ELISA	FCS; BSA	Trypsin; antibodies	Animal sera in trypsin neutralising solution	29,30
Human keratinocytes cell line NCTC2544	Production of IL-18	Sandwich ELISA	FCS	Antibodies	Animal sera in trypsin neutralising solution; animal-derived blocking agent	31–35
HaCaT cell line	Gene profiling	Microarray	FCS	Trypsin	Animal sera in trypsin neutralising solution	25
AREc32 human cell line and Hepa1C1C7 murine cell line	Activation of the Keap/Nrf2/ARE regulatory pathway	Luciferase and quinone-reductase activity	FCS; BSA	—	Trypsin and animal sera in trypsin neutralising solution	36
Human keratinocyte cell line harbouring a luciferase reporter gene under control of an ARE-element	Induction of genes controlled by the ARE	Luciferase activity (by using a luminometer)	FBS	Trypsin	Animal sera in trypsin neutralising solution	37
Transgenic cell line with a luciferase reporter gene under control of an ARE-element (KeratoSens)	Activation of the ARE-Nrf2 pathway	Luciferase activity by using a luminometer	FCS ^d	Trypsin ^d	Trypsin ^d and animal sera in trypsin neutralising solution ^d	38–43
HaCaT cell line	Expression of genes controlled by response elements	qRT-PCR	FBS	—	Trypsin and animal sera in trypsin neutralising solution	44

K: keratinocytes; FBS: fetal bovine serum; IL: interleukin; PCR: polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; FCS: fetal calf serum; BSA: bovine serum albumin; Keap1: Kelch-like ECH-associated protein 1; Nrf2: nuclear factor-erythroid 2-related factor 2; ARE: antioxidant response element; qRT-PCR: quantitative real-time polymerase chain reaction.

^aAnimal-derived products that were clearly mentioned in the paper.

^bPotentially animal-derived products that were mentioned in the paper — that is, when the products that were mentioned are usually animal-derived, but no specific information was provided regarding their source in this particular case.

^cAnimal-derived products that were probably used, according to the technique, but were not actually mentioned in the paper.

^dNot used in the KeratoSens adapted to animal product-free cell culture (Belot et al.).³⁸

Table 2. Details of the products of animal origin likely to have been used in the 50 analysed *in vitro* methods for assessing the response of dendritic cells.

Platform	Read-out parameter	Technique	Animal-derived products ^a	Potentially animal-derived products ^b	Probable animal-derived products ^c	References
DC generated from human PB	IL-1 β gene expression	RT-PCR and flow cytometry	FCS; BSA	Antibodies	Animal-derived blocking agent	45–47
DC generated from human PB	Alteration of HLA-DR expression	Flow cytometry	FCS; BSA	Collagen; antibodies	—	48
Human DC derived from human PB mononuclear cells	Modulation of receptor-mediated endocytosis	Flow cytometry	FCS	Antibodies	—	49
Freshly isolated epidermal LC from adult human skin	Pattern of HLA-DR endocytosis	Laser scanning confocal and electron microscopy	—	Trypsin; antibodies	Animal sera in trypsin neutralising solution	50
Epidermal LC from mice ears and pelts	Formation of phosphotyrosine in murine epidermal LC	Flow cytometry	FCS	Trypsin; antibodies	Animal-derived blocking agent	51,52
Mononuclear cells from human PB	Secretion of IL-1 α	ELISA	FCS; BSA	Antibodies	Animal-derived blocking agent	53
Monocyte-derived DC-like cells from human donors	Activation of immature DC by CD86, IL-1 β and aquaporin P3 gene expression	Flow cytometry and q-RT-PCR	FCS	Antibodies	Animal-derived blocking agent	54
Monocyte-derived immature DC from human PB	Changes in surface markers expression	Flow cytometry	FCS	Antibodies	Animal-derived blocking agent	55
Immature DC derived from human monocytes (PB)	Expression of chemokine receptors and surface markers	Flow cytometry	FCS	Antibodies	Animal-derived blocking agent	56
Immature DC derived from human monocytes (PB)	Upregulation of CD86	Flow cytometry	FBS; BSA	Antibodies	—	57
Human monocyte-derived DC from PB	Increase in the expression of CD54, CD86 and HLA-DR	Flow cytometry	FCS; BSA; rabbit serum	Antibodies	—	58,59
Immature DC isolated from mononuclear human cells (PB)	Upregulation of CD86, CD54 and HLA-DR	Flow cytometry	FCS	Trypsin; antibodies	Animal-derived blocking agent	60
Human monocyte-derived DC from PB	Induction of CD86 and HLA-DR expression	Flow cytometry	FBS	Antibodies	Animal-derived blocking agent	61
CD34 ⁺ progenitor-derived DC from human cord blood	Upregulation of CD86, CD83 and HLA-DR	Flow cytometry	FBS; BSA	Heparin; antibodies	Animal-derived blocking agent	62
CD34 ⁺ progenitor-derived DC from human cord blood	Expression of CCL2, CCL3 and CCL4 chemokine genes	Flow cytometry and qRT-PCR	FBS; BSA	Antibodies	—	63
Human PB monocyte-derived DC (CD1a ⁺ / CD14 ⁺)	Expression of CD86	Flow cytometry	FCS	Antibodies	Animal-derived blocking agent	64
CD34 ⁺ progenitor-derived DC from human cord blood	Transcriptome analysis of 13 genes	qRT-PCR	FCS and BSA	Sodium heparin; antibodies	—	65,66
DC generated from human mononuclear cells (CD14 ⁺ monocytes)	Activation of p38 MAPK and regulation of MAPK signalling pathways	Fast activated cell-based ELISA (Face TM) and cytometric bead assay (Phosphoflex TM)	FBS	Antibodies	—	67
CD34 ⁺ progenitor-derived DC from human cord blood	Changes in gene expression	Flow cytometry, whole genome microarray, immune toxicity chip	FBS; BSA	Antibodies	Animal-derived blocking agent	68–70
CD34 ⁺ progenitor-derived DC from human cord blood	Cell damage and changes in the expression of CREM and CCR2 genes	Cytotoxicity assays and qRT-PCR	FBS; BSA	Sodium heparin; antibodies	—	71

(continued)

Table 2. (continued)

Platform	Read-out parameter	Technique	Animal-derived products ^a	Potentially animal-derived products ^b	Probable animal-derived products ^c	References
Peripheral blood-derived DC from commercial preparation or immature DC derived from human monocytes	Changes in the expression of 10 genes	qRT-PCR and flow cytometry	FBS	—	—	72
Monocyte-derived DC isolated from leukocyte-enriched buffy coats from different donors	Production of CXCL8 (IL-8)	ELISA	FCS; BSA	Antibodies	Animal-derived blocking agent	73
Monocyte-derived DC isolated from leukocyte-enriched buffy coats from healthy donors	Expression of a set of genes	Low-density cDNA microarray (oligo sorbent array)	—	Antibodies	—	74
THP-1 cells	Expression of CD86 and internalisation of MHC class II molecules	Flow cytometry	FBS; BSA	Antibodies	—	75
THP-1 cells	Upregulation of MIP-1 β and CD86 expression	Oligo-DNA microarray and flow cytometry	FBS; BSA	Antibodies	—	76
THP-1 cells	Expression of CD40, CD83, CD86 and CD54; and secretion of TNF- α and IL-8	Flow cytometry and ELISA	FBS; BSA	Antibodies	Animal-derived blocking agent	77
THP-1 cells	IL-8 production	ELISA	FBS or FCS	Antibodies	Animal-derived blocking agent	78,79
THP-1 cells	Production of macrophage inflammatory protein 1- β	Sandwich ELISA	FBS	Antibodies	Animal-derived blocking agent	80
THP-1 cells	Transcriptional response of 13 genes	qRT-PCR	FBS	—	—	81
THP-1 cells	Expression of CD54 and CD86	Flow cytometry	FBS ^{de}	Antibodies ^d	—	82,83–85,86–91
THP-1 cells	Expression of CD54, CD86 and CD40	Flow cytometry	FBS	Antibodies	—	92
Mature DC derived from THP-1 cells	Changes of cell surface thiols	Flow cytometry	FBS	Antibodies	Animal-derived blocking agent	93
THP-1 cells	Expression of IL8, HMBOX1 and PAI1 genes	qRT-PCR	FBS	—	—	94
THP-1 derived cell line THP-G8 (stable IL-8 reporter)	Induction of IL-8 messenger RNA (IL-8 Luc assay)	Luciferase activity by using a microplate-type luminometer	FCS	—	—	95
MUTZ-3 cells	Expression of CD86	Flow cytometry and microarray	FBS	Antibodies	Trypsin and animal sera in trypsin neutralising solution	96
MUTZ-3 cells differentiated into LC	Migration of MUTZ-LC and CXCL8 secretion	ELISA, flow cytometry, and transwell migration assay (by using fluorescence staining)	FCS; BSA	Antibodies	Animal-derived blocking agent	97
MUTZ-3 cells	Expression of programmed death receptor ligand-1 (PDL-1)	Flow cytometry	FCS; BSA	Antibodies	—	98
MUTZ-3 cells (GARD assay)	Gene signature	Flow cytometry	FCS; BSA	Antibodies	—	99–101
MUTZ-3 cells differentiated into immature MUTZ-LC	Migration of MUTZ-LC towards the chemokines CXCL12 and CCL5	Flow cytometry and transwell migration assay	FCS; BSA	Trypsin; antibodies	Animal-derived blocking agent	102,103
Human epidermal LCs (human skin from plastic surgery)	E-cadherin and HLA-DR levels	Flow cytometry	FCS	Trypsin; antibodies	—	104

(continued)

Table 2. (continued)

Platform	Read-out parameter	Technique	Animal-derived products ^a	Potentially animal-derived products ^b	Probable animal-derived products ^c	References
Ex vivo human skin explant cultures	Induction of CD1a ⁺ LC migration	Immunohistochemistry	Normal rabbit serum; BSA	Antibodies	—	105
Mouse bone marrow-derived DC	Expression of DC-related markers and cytokines	Flow cytometry and RT-PCR	Bones (tibiae and femurs) from mice	Antibodies	Animal sera in 'complete medium'; animal-derived blocking agent	106
Human myeloid cell line U937	Expression of CD86 (and apoptosis)	Flow cytometry	FBS or FCS	Antibodies	—	107,108
Human myeloid cell line U937	Expression of CD86, IL-1 β and IL-8	Flow cytometry, qRT-PCR and ELISA	FCS	Antibodies	—	109
Co-culture of the cell lineages THP-1 (DC-like) and HaCaT (K)	Expression of CD40, CD54 and CD86	Flow cytometry	FBS	Trypsin; antibodies	Animal-derived blocking agent	110
Co-culture of the cell lineages THP-1 (DC-like) and HaCaT (K)	Expression of CD54 and CD86	Flow cytometry	FBS	Antibodies	Trypsin, animal sera in trypsin neutralising solution; animal-derived blocking agent	111
THP-1 (DC-like) cells co-cultured with primary human K	Expression of CD54 and CD86	Flow cytometry	FBS; trypsin; bovine pituitary extract; bovine transferrin; BSA	Antibodies	Animal sera in trypsin neutralising solution	112
Human K co-cultured with allogenic monocytes	Expression of CD86	Fluorescence activated cell sorting (FACS)	FCS	Trypsin; antibodies	Animal sera in trypsin neutralising solution; BSA	113,114
DC-related cells (from PB) co-cultured with primary human K (from human skin)	Expression of CD86 and production of IL-6 and MIP-1 β	Flow cytometry and ELISA	FCS	Trypsin; antibodies	Animal sera in trypsin neutralising solution; animal-derived blocking agent	115
Murine dendritic cell line X552	Expression of MHC class II molecules	Flow cytometry	Murine serum; FCS	Antibodies	—	116

DC: dendritic cells; PB: peripheral blood; IL: interleukin; RT-PCR: reverse transcriptase polymerase chain reaction; FCS: fetal calf serum; BSA: bovine serum albumin; HLA-DR: human leukocyte antigen DR isotype; LC: Langerhans cells; ELISA: enzyme-linked immunosorbent assay; qRT-PCR: quantitative real-time polymerase chain reaction; FBS: fetal bovine serum; CREM: cyclic adenosine monophosphate-responsive element modulator; CCR2: monocyte chemoattractant protein-1 receptor; MHC: major histocompatibility complex; MIP: macrophage inflammatory protein.

^aAnimal-derived products that were clearly mentioned in the paper.

^bPotentially animal-derived products that were mentioned in the paper — that is, when the products that were mentioned are usually animal-derived, but no specific information was provided regarding their source in this particular case.

^cAnimal-derived products that were probably used, according to the technique, but were not actually mentioned in the paper.

^dNot used in the h-CLAT adapted to animal product-free conditions (Edwards et al.).⁸²

^eNot used in the h-CLAT adapted to chemically defined culture (Marigliani et al.).⁸³

Table 3. Details of the products of animal origin likely to have been used in the eight analysed *in vitro* methods for assessing T-cell response.

Platform	Read-out parameter	Technique	Animal-derived products ^a	Potentially animal-derived products ^b	Probable animal-derived products ^c	References
Naïve T-cells and chemical modified/pulsed monocyte-derived DC co-cultured with a CD3-feeder layer	Frequency of T-cells and antigen-specific production of IFN- γ and TNF- α	Flow cytometry	—	Antibodies	—	117
DC/LC from normal human skin cultured with autologous T-cells isolated from HPB	Hapten-specific proliferation of T-cells	³ H-thymidine incorporation assay by using a scintillation counter and/or flow cytometry	FCS ^d	Trypsin; antibodies	Animal sera in trypsin neutralising solution	118–120
DC derived from mononuclear cells from HPB co-cultured with autologous T-cells	Autologous proliferative T-cell response	Flow cytometry and ³ H-thymidine incorporation	FCS; sheep red blood cells	Antibodies	—	121
CD34 ⁺ hematopoietic progenitor cells from cord blood; T-cells from HPB or cord blood	T-lymphocytes priming by DC	Flow cytometry; FACS and ³ H-thymidine incorporation	FCS; sheep erythrocytes; BSA	Antibodies	—	122
Immature LC-like DC from cord blood and autologous T-lymphocytes	Phenotypic maturation of DC and lymphocyte activation	Flow cytometry and ³ H-thymidine incorporation	FCS; sheep erythrocytes; BSA	Antibodies	—	123
Monolayered Pam 212 cells (K) co-cultured with T-cells and macrophages from non-sensitised mice	Activation of hapten-specific T-cells (proliferation)	³ H-thymidine incorporation	T-cells from spleens of female Balb/c mice; FCS; BSA	Antibodies	Trypsin and animal sera in trypsin neutralising solution	124,125
Haptenated monocyte-derived DC co-cultured with CD25 ⁺ cell-depleted lymphocytes (all from HPB)	T-cell priming to haptens by cell proliferation and production of IFN- γ	³ H-thymidine incorporation and ELISA	FCS	Antibodies	Animal-derived blocking agent	126
T-cells and monocytes isolated from peripheral blood (donors)	Specific T-cell proliferation and IFN- γ production in CD4 ⁺ and CD8 ⁺ T-cell subsets	³ H-thymidine incorporation and flow cytometry	—	Antibodies	—	127

DC: dendritic cells; LC: Langerhans cells; INF: interferon; TNF: tumour necrosis factor; FCS: fetal calf serum; HPB: human peripheral blood; FACS: fluorescence activated cell sorting; BSA: bovine serum albumin; FBS: fetal bovine serum; ELISA: enzyme-linked immunosorbent assay.

^aAnimal-derived products that were clearly mentioned in the paper.

^bPotentially animal-derived products that were mentioned in the paper — that is, when the products that were mentioned are usually animal-derived, but no specific information was provided regarding their source in this particular case.

^cAnimal-derived products that were probably used, according to the technique, but were not actually mentioned in the paper.

^dFetal calf serum only mentioned in Rustemeyer et al.¹²⁰

Table 4. Details of the products of animal origin likely to have been used in the 17 analysed *in vitro* methods involving reconstructed epidermis, skin models or explants.

Platform	Read-out parameter	Technique	Animal-derived products ^a	Potentially animal-derived products ^b	Probable animal-derived products ^c	References
Reconstructed human epidermis (EPISKIN TM) made of human-derived epidermal K in monolayer	Induction of IL-12p40	Semi-quantitative RT-PCR and ELISA	—	Antibodies	Trypsin and animal sera in trypsin neutralising solution; BSA in ELISA buffer	128
Layers of differentiated human K (EpiDerm TM , MatTek Corporation)	Damage, viability and release of cytokines and proinflammatory mediators	Histology, MTT assay and ELISA	—	Antibodies	Trypsin and animal sera in trypsin neutralising solution; animal-derived blocking agent	129
Reconstructed human epidermis (normal human K)	Cell morphology, intracellular and extracellular levels of IL-1 α and IL-8	Immunohistochemistry; ELISA	—	Antibodies	Trypsin and animal sera in trypsin neutralising solution; BSA in ELISA buffer	130
Reconstructed human epidermis obtained by culturing normal human K (SkinEthic)	Differential IL-1 α and IL-8 synthesis and release	Quantitative sandwich immunoassay technique (ELISA) and RT-PCR	—	Antibodies	Trypsin and animal sera in trypsin neutralising solution; animal-derived blocking agent	131
Reconstructed human epidermis obtained by culturing epidermal K isolated from neonatal foreskins	Cell viability and IL-1 α secretion for sensitising potency	MTT assay and ELISA	—	Antibodies	Trypsin and animal sera in trypsin neutralising solution; animal-derived blocking agent	132
Human reconstituted skin models (EpiDerm TM (MatTek) and SkinEthic) generated by using normal human K	Activation of the Nrf2/ARE expression pathway	qRT-PCR	—	—	Trypsin and animal sera in trypsin neutralising solution; animal-derived blocking agent	133
Reconstructed human epidermis EpiDerm TM (MatTek) using 24- and 96-well formats	Epidermal Sensitisation Assay (EpiSensA): expression of ATF3, DNAB4 and GCLM genes	qRT-PCR	—	—	Trypsin and animal sera in trypsin neutralising solution	134
Reconstructed human epidermis containing LC (CD34 ⁺ -derived DC co-seeded with heterologous K onto EpiSkin support)	Reactivity of integrated LC (number, morphology, IL-1 β and CD86 over expression)	Immunohistochemistry and RT-PCR	FCS	Antibodies	Trypsin and animal sera in trypsin neutralising solution	135,136
In-house and commercially available reconstructed human epidermis models	IL-18 release (potential) and cytotoxicity (potency)	MTT assay and ELISA	—	Antibodies	Trypsin and animal sera in trypsin neutralising solution; animal-derived blocking agent	137

(continued)

Table 4. (continued)

Platform	Read-out parameter	Technique	Animal-derived products ^a	Potentially animal-derived products ^b	Probable animal-derived products ^c	References
Reconstructed human epidermis model (Episkin/SENS-IS)	Gene expression pattern	qRT-PCR	—	—	Trypsin and animal sera in trypsin neutralising solution	138,139
Reconstructed full thickness skin model (RealSkin) co-cultured with MUTZ-3 derived LC	Secretion of IL-12, IL-9, VEGF and IFN- γ	Flow cytometry and cytokine multiplex assay	FBS; mouse serum	Antibodies; collagen	Trypsin and animal sera in trypsin neutralising solution; animal-derived blocking agent	140
Reconstructed epidermis by using a human K cell line (N/TERT)	Activation of the Keap1-Nrf2-ARE pathway	Immunohistochemistry; immunofluorescence; Western blotting; nuclear fractionation assay; qRT-PCR	Bovine pituitary extract; BSA; goat serum; dried skimmed milk	Antibodies	Trypsin and animal sera in trypsin neutralising solution	141
Cultures of skin biopsies from healthy donors	Migration of LC	Immunohistochemistry	BSA	Antibodies	—	142
Human skin biopsies cultured with autologous primed lymphocytes	Histological damage	Histopathological evaluation	FCS	—	—	143
Skin model containing collagen vitrigel membrane, DC, K and fibroblasts	Expression of CD86 and cytokine release (IL-1 α and IL-4)	Immunostaining and enzyme immunoassay	Type I collagen from fish scale gelatin (tilapia) (Nitta Gelatin) and from bovine derm is (Koken Co.); FBS; normal goat serum	Antibodies	Trypsin and animal sera in trypsin neutralising solution; animal-derived blocking agent	144,145
Skin equivalent containing K, fibroblasts and melanocytes; and human LC derived from MUTZ-3 cells	Maturation and migration of epidermally incorporated LC	Immunohistochemistry; flow cytometry; qRT-PCR	FCS	Antibodies; collagen gels	Trypsin and animal sera in trypsin neutralising solution; animal-derived blocking agent	146
Murine and human skin explants; and reconstituted skin models (epidermal model EST-100 and full-thickness model AST-2000)	Activation (phosphorylation) of MAP-kinases (p38, ERK1/2 and JNK1/2)	Cytometric bead array	Murine skin explants from 9–10 week-old female hairless C3H/TifBom hr/hr mice	Antibodies	Trypsin and animal sera in trypsin-neutralising solution; animal-derived blocking agent	147

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; K: keratinocytes; ELISA: enzyme-linked immunosorbent assay; IL: interleukin; RT-PCR: reverse transcriptase polymerase chain reaction; LC: Langerhans cells; VEGF: vascular endothelial growth factor; IFN: interferon; qRT-PCR: quantitative reverse transcriptase polymerase chain reaction; BSA: bovine serum albumin; FBS: fetal bovine serum; FCS: fetal calf serum.

^aAnimal-derived products that were clearly mentioned in the paper.

^bPotentially animal-derived products that were mentioned in the paper — that is, when the products that were mentioned are usually animal-derived, but no specific information was provided regarding their source in this particular case.

^cAnimal-derived products that were probably used, according to the technique, but were not actually mentioned in the paper.

possible to identify the origin of the heparin (porcine mucosa, according to the manufacturer's website). However, for most of the methods that employed human blood derivatives, it was not possible to identify the origin of the heparin used.

Several other animal-derived products were identified, such as bovine pituitary extract, collagen and dried skimmed milk. The main products used are discussed below, with their respective ethical and technical drawbacks, and some examples of available alternatives.

Discussion

Animal sera

Animal sera, mainly FBS, was the most common animal-derived product used in the methods described in our selected sample of articles. FBS is an important source of amino acids, vitamins, trace elements, hormones, growth factors and other elements.¹⁴⁸ Although it is routinely included as a culture medium supplement (and also in various cell freezing media, at a concentration of up to 90% v/v), its use has been highly criticised for ethical reasons (see 'Introduction' section).¹⁹ Furthermore, alongside its high cost,¹⁴⁹ the use of animal serum also has scientific implications,²² as it represents a potential source of contaminants and its undefined composition and high inter-batch variability can lead to inconsistency between experiments.^{150,151} Due to its high complexity, serum composition is inherently ill-defined.¹⁵² Batch-to-batch variability is attributed to the animal's diet, and geographical and seasonal variations,^{153,154} which can cause quantitative and qualitative variation in cell growth and differentiation responses.^{155,156}

The elimination of animal sera from protocols could greatly improve the quality of *in vitro* methods, which in turn would have a positive impact on within-laboratory and between-laboratory reproducibility, which are major concerns in terms of validation and regulatory acceptance.¹⁵¹ Several FBS substitutes exist, including other animal-derived products, such as newborn and adult bovine sera, sera from other species (e.g. horse, pig and goat), tissue extracts and bovine milk fractions.¹⁴⁸ Also, plant extracts¹⁵⁷ and platelet-derived products¹⁵⁸ are available. Human platelet lysate is a promising alternative,¹⁵⁹ as is human serum, which has already been used in validated alternative methods for skin sensitisation.^{38,82} These two methods^{38,82} use commercial human plasma (pooled human male AB plasma) as the source, which is more defined than animal serum but is still 'ill-defined'. As such supplements are human-derived and contain unidentified trace elements and unknown factors, their availability is limited and they pose the risk of human pathogen transmission.¹⁶⁰ The implications in terms of inter-batch variability must be assessed for each specific method. As the

harvesting of plasma requires the use of an anticoagulant, to be truly free of animal-derived products, the blood harvesting and pooling process (for human serum or platelet-derived products) must not use animal-derived anticoagulants. For example, some commercially available human platelet lysates are not completely free of animal products because they use porcine-derived heparin in their preparation.¹⁶¹ However, there are 'animal-free' anticoagulants that could be used, such as citrate.^{38,82} Concerns regarding animal-derived heparin also apply to all *in vitro* methods that use blood-derived cells.

In order to avoid the use of FBS, a range of media can be employed that are classified as 'serum-free' or 'chemically defined'. However, it should be noted that 'serum-free' media often contain other animal-derived components, such as albumin, hormones and growth factors.¹⁶² Therefore, to ensure that cell culture methods are completely free of animal-derived products, it is necessary to use chemically defined media that are certified as animal product-free.

Purified proteins can be of human, animal and non-animal origin, but the risk of viral contamination remains whenever human-derived or animal-derived substances are used.¹⁶³ Recombinant proteins expressed in bacteria, yeast or animal cells can be used as synthetic media supplements.¹⁶⁴ For some commercially available RSE models, there is information on the culture medium used for their production — for example, serum-free medium is used by MatTek for the EpiDerm™ model.¹³³ However, in this case, there is no information available on the source of growth factors and hormones used, nor specific indication that it is an animal origin-free method.

The use of chemically defined media is part of Good In Vitro Method Practice (GIVIMP),¹⁶⁵ which is an attempt to increase the reliance of *in vitro* methods by developing a common standard.¹⁵⁴ Nowadays, a growing variety of chemically defined media are commercially available, which also includes tailor-made options. In addition, a serum-free media interactive online database is available open access (<http://fcs-free.org>).¹⁴⁸

The EURL European Centre for the Validation of Alternative Methods (EURL ECVAM) Scientific Advisory Committee has endorsed the use of non-animal-derived products and stated that, for validation purposes, when the use of non-animal alternatives to serum is not fulfilled, a justification should be provided "including measures taken to seek non animal alternatives" (see the OECD, *Guidance Document on Good In Vitro Method Practices*).¹⁶⁵

Despite the high use of FBS found in this study, a recent trend to reduce or eliminate it in some methods was noticed. The first version of OECD Test Guideline (TG) 442D (ARE-Nrf2 Luciferase Test Method)¹⁶⁶ featured only a KeratinoSens™-based method in which cells were cultured in medium supplemented with 9% FCS.⁴² The latest version,³⁹ which includes LuSens,³⁷ states that

KeratinoSens culture “may be performed using human reagents (human serum and recombinant human trypsin) to achieve xeno-free cell culture”.

The use of chemically defined media has increasingly been recommended to circumvent the drawbacks of animal and human sera,¹⁶⁵ and the successful adaptation of THP-1 cells to chemically defined culture conditions has been reported.⁸³ The three methods outlined in OECD TG 442E⁸⁴ — that is, human Cell Line Activation Test (h-CLAT),⁸⁵ U-SENSTM¹⁰⁷ and the IL-8 Luc assay⁹⁵ — all include the use of animal-derived products. However, with regard to the h-CLAT, it is a promising development that THP-1 cells are used in this test, and also that the method has recently been adapted to animal product-free conditions by using human serum, human albumin and synthetic antibodies.⁸²

When planning to replace animal serum, appropriate shear-stress protection for the cells should be considered. This is a necessary consideration, which is essential not only in large-scale manufacturing processes that involve stirring but also during seemingly trivial pipette manipulations in the cell culture laboratory. FBS contains various components, mainly albumin, that protect cells from shear stress, but the non-ionic surfactant Pluronic[®] F68 is a commonly used alternative that provides similar protection.^{167,168}

Bovine serum albumin

BSA is a highly stable globular polypeptide chain isolated from bovine blood, often fetal bovine blood (therefore, with the same ethical implications as FBS). It is used in several biochemical techniques as a stabiliser for other solubilised proteins and is considered a universal blocking agent in various immunological techniques.^{169,170}

When working with antibodies, the prevention of non-specific binding is routinely achieved through the use of an animal serum or albumin, or other animal-derived blocking agent, such as dried skimmed milk, casein, whole normal serum or fish gelatin.¹⁷¹ Therefore, although the use of BSA was mentioned in some of the analysed immunological protocols, the use of animal-derived products for this purpose was highly probable, even if it was not specifically mentioned.

Despite the extensive use of BSA in immunological methods, several non-animal alternative products exist, including detergents, polymers,¹⁷¹ plant proteins (such as lectins)¹⁷² and human serum albumin.^{82,117}

Trypsin

Enzymes are often used to dissociate tissue and to detach adherent cells prior to subculturing. Trypsin, usually derived from the pancreas of pigs, is the most commonly available enzyme for such purposes.¹⁷³ Trypsin was mentioned in 62% of the methods identified to assess keratinocyte activation, but only one method — the DB-ALM protocol for KeratinoSens¹⁷⁴ in OECD TG 442E⁸⁴ —

clearly mentioned the manufacturer (i.e. Gibco) and catalogue number, from which it could be confirmed that it was derived from porcine pancreas. For the other four methods of this type, it was not possible to identify the source of the trypsin or the manufacturer. Thus, the trypsin was classified as ‘potentially animal-derived’.

The use of animal-derived trypsin was clearly described in only one of the 50 methods to assess DC response, in which it was used to dissociate keratinocytes from foreskin.¹¹² As the trypsin manufacturer (Invitrogen Corp., now ThermoFisher Scientific) was mentioned in this case, it was possible to identify its porcine origin. Whenever trypsin was used for the isolation of cells from epidermal human or animal skin,^{50,51} or for subculturing primary human keratinocytes or keratinocyte lineages in co-culture with DCs,^{111,112} most of the analysed methods did not state the source or the manufacturer. Therefore, the trypsin was again classified as ‘potentially animal-derived’.

Although trypsin was not mentioned in the many of the papers analysed, its use was likely, in that most of the methods involved adherent cells (e.g. keratinocytes and fibroblasts), even if only as feeder layers. In addition, trypsin is part of standard laboratory protocols for the disaggregation of tissues derived from humans or animals. Therefore, trypsin was classified as an ‘animal-derived product probably used’ in all methods that mentioned the above procedures and did not specify the use of another product. It was also likely that animal-derived trypsin would have been used in a number of other methods, for example, for subculturing the cells used to prepare conditioned medium,⁹⁶ or in the manufacturing of commercially available reconstructed human epidermis.^{128,129}

In addition to animal welfare issues, trypsin can also constitute a source of contamination, especially by mycoplasma.¹⁷⁵ However, trypsin and other such proteases can be obtained from a variety of sources: invertebrates (e.g. Accutase[®]), bacteria (e.g. collagenase, dispase, pronase), humans (recombinant human trypsin³⁸) and plants (e.g. TrypLETM, TrypZean[®]).¹⁷⁶

After detaching cells with trypsin, it is necessary to stop its action (‘neutralisation’). This is usually performed by the addition of animal serum (such as FBS), and it is such a standard process when subculturing adherent cells that this step is not usually mentioned in the method. The use of an animal serum such as FBS for the neutralisation of trypsin has the same ethical and technical implications as its use as medium supplement. Importantly, it should be noted that a further advantage of the non-animal-derived replacements for trypsin is that they do not require neutralisation with animal serum.

Primary cells of non-human origin

Some of the analysed methods to assess keratinocyte activation were based on murine cell lines,²⁹ but primary

animal cells such as skin cells obtained from the backs and ears of BALB/c mice were also used.²⁸ One of the methods to assess the T-cell response used murine cells — specifically, a keratinocyte cell line (Pam 212) and T-cells and macrophages from non-sensitised BALB/c mice.^{124,125}

According to the Three Rs principles (*Replacement, Reduction, Refinement*), first put forward by Russell and Burch in 1959,¹⁷⁷ primary cells and tissues from non-human animals are a partial replacement. From a scientific point of view, in order to ensure that *in vitro* methods mimic the ‘real-life’ response, the full replacement of animal cells and tissues with human cells (primary cells or cell lines) and donated human tissues is desirable.

Antibodies

Since the development of the technology in the 1970s,¹⁷⁸ monoclonal antibodies have been produced by hybridomas, which are myeloma cells fused with B-lymphocytes from immunised animals. These hybridomas can be propagated *in vivo* in ascites fluid (by injecting hybridomas into the peritoneal cavity of animals to produce an antibody-rich fluid as a response) or *in vitro* by culturing hybridoma cells in culture flasks or bioreactors. Despite producing a higher concentration of antibodies, the ascites method has many disadvantages,¹⁷⁹ including considerable animal pain and distress.¹⁸⁰ In fact, it has been phased out in many countries. The propagation of hybridomas *in vitro* constitutes a *refinement*, but besides animal welfare considerations, the production of antibodies by animal-based methods — either directly (in the discovery phase) or indirectly (by using FBS in the production phase) — also presents a number of scientific issues, such as the risk of contamination and lot-to-lot variability.¹⁸¹

We identified a high level of antibody use in the analysed articles, as most of the methods included immunological techniques that rely on monoclonal and/or polyclonal antibodies. Some of the methods employed several antibodies from different manufacturers and species.⁶⁰ While the manufacturers of the antibodies were sometimes mentioned, the production techniques were not, which made it difficult to determine whether they involved animals and/or animal-derived products. Therefore, when there was no clear information on the production technique, the antibodies were considered ‘potentially animal-derived products’, in view of the fact that most traditional methods use animals.

Currently, high yields of antibodies can be produced *in vitro*.^{182–184} Despite the relatively high cost and long culture periods, mammalian cells have been the major host, mainly due to their capacity for correct protein glycosylation and extracellular secretion. Recent advances in antibody engineering employ simpler cost-effective hosts as bioreactors, such as yeast and bacteria (especially for monoclonal antibody fragments)¹⁸⁵ and plants.¹⁸⁶ It is now possible to circumvent the limitations (and ethical

implications) of animal-derived antibodies by using recombinant antibodies¹⁸¹ and fully *in vitro* techniques with the advent of synthetic antibodies.¹⁸⁷ Custom antibodies produced by phage-display technology have already been shown to successfully replace animal-derived antibodies in the h-CLAT.⁸²

Other animal-derived products

The use of animal-derived products in non-animal alternative methods seems a contradiction in terms, but in fact some *in vitro* methods actually use several or more. For example, one experimental study involved the use of bovine pituitary extract, BSA, goat serum, dried skimmed milk and antibodies.¹⁴¹ In some cases, the origin of the products was unclear: for instance, Cao et al.¹¹² mentioned the use of a human keratinocyte growth supplement, which, after checking the manufacturer’s website, was found to include animal-derived bovine pituitary extract and transferrin.

Other examples include the use of sheep erythrocytes to purify T-cells,^{121–123,188} and the use of collagen, which was mentioned by three of the 16 methods involving RSE and related models. Although significant efforts have been made to produce recombinant non-animal collagen,¹⁸⁹ unless otherwise stated, it was assumed that any collagen used in the analysed methods was of animal origin due to its lower cost. Only one method¹⁴⁴ mentioned the manufacturers of the collagens used — further investigation showed that type-I collagen (from Nitta Gelatin, Osaka, Japan) can be derived from porcine, bovine or fish raw materials, while collagen vitrigel membrane (from Koken Co., Tokyo, Japan) is prepared from bovine dermis, according to their respective websites.

Anticoagulants comprise another group of products whose animal origin might not be particularly obvious. Some methods that used human cord blood to generate progenitor-derived cells stated that the blood collection procedure was performed with collection tubes containing sodium heparin as an anticoagulant. When the manufacturer was mentioned,^{62,65} it was possible to identify the origin of the heparin (porcine mucosa). Heparin (which is usually animal-derived) tends to be used in methods involving animal or human blood collection, as it is often preferred for this purpose over other anticoagulants such as ethylenediaminetetraacetic acid (EDTA).¹⁹⁰ When working with human blood derivatives, it is necessary to investigate the method chosen for blood collection, as animal-derived heparin is commonly used,¹⁹¹ although animal-free anticoagulants are available.^{38,82}

Reflections on cruelty-free cosmetics

Some animal-derived products are considered to be by-products of the meat industry. Although their origin does

not directly imply animal suffering per se (as is the case for FBS), and the tissues (such as viscera) would probably be discarded, they are derived from animals raised for human consumption, which implies some extent of inherent animal suffering.

It is believed that the worldwide demand for FBS will not be met by fetuses taken from cows that (by chance) arrive pregnant at the slaughterhouse. It is actually a highly profitable market, which produced around 500,000 litres of FBS per year in the 1990s, from one to two million bovine fetuses.¹⁹ The global sales volume of FBS surpassed 650,000 litres in 2017, and the global market was valued at US\$970 million in 2018.¹⁹² Therefore, FBS should not be considered to be a ‘by-product’. If FBS, or any so-called animal ‘by-product’, is used in an alternative *in vitro* method, then it should not be referred as animal-free. Importantly, it seems that cosmetics can be mistakenly referred to as ‘cruelty-free’, even when their ingredients have been tested by using *in vitro* methods that make use of such animal-derived products.

Animal-derived products have been used in animal cell culture since its development at the beginning of the 19th century. However, they are gradually being replaced by products of synthetic origin.¹⁶⁴ Russell and Burch defined *replacement* as the use of non-sentient material instead of a sentient being, and *absolute replacement* as when “animals are not required at all at any stage”.¹⁷⁷ Although, according to this definition, *in vitro* methods for the safety assessment of chemicals are *replacement* methods, this review shows that, in most cases, the *replacement* is only partial. In our opinion, historical reasons and a lack of public awareness regarding ethical issues related to these animal-derived products — and a consequent lack of demand for changes — have hindered *absolute replacement* in this field. However, in order to increase human-relevance, and also due to animal welfare issues, there has been a recent cosmetic industry requirement to eliminate animal-derived products from *in vitro* methods. As non-animal alternatives to these products provide ethical and technical advantages, this issue should be further discussed by the scientific community, funding agencies and policymakers, in order to encourage a full replacement of animal use in safety assessment.

Conclusions

The use of animal-derived products (mainly FBS, BSA, trypsin and antibodies) in methods developed to replace the use of animals in skin sensitisation testing is highly prevalent. Given the conflicting nature of this situation, some recent efforts have been made to adapt some alternative *in vitro* methods to exclude the use of animal (i.e. non-human)-derived products.^{38,82,83}

It was clear from this review that not all of the likely animal-derived products used were mentioned in the

methods. Even when they were mentioned, in most cases, it was not clear whether they were of animal origin. As the use of animal-derived products brings a number of drawbacks to *in vitro* methods, and also works against their primary aim — that is to reduce and, preferably, eliminate animal suffering — published papers should mention every animal-derived product that is used in an *in vitro* method. Conversely, when no animal-derived products are used, this should be explicitly stated.

As most of the *in vitro* methods analysed in this review involved the use of animal-derived products, it can be implied that many beauty products are not as ‘cruelty-free’ as some cosmetic companies claim. The identification of animal-derived products draws attention to this issue and calls for efforts to replace these products in existing *in vitro* methods and to avoid them in the development of new ones, in order to provide technically and ethically better methods for safety assessment.

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