Ersatzes of Fetal Bovine Serum: a survey of current options in cell culture: Example of the Regenerative Therapy Unit/CPR/CHUV

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Abbreviations

Allo-HS	Human allogenic serum
ASCs	Adipose derived stromal cells
AT-MSC	Adipose tissue derivedmesenchymal stromal cells
Auto-HS	Human autologous serum
BM-MSC	Bone marrow derived mesenchymal stromal celles
BMSCs	Bone marrow derived stromal cells
CPC	Centre de Production Cellulaire
EMA	European Medicines Agency
EUTCD	European Tissue and Cell Directive
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GMP	Good Manufacturing Practice
hMSCs	Human mesenchymal stromal cells
HPL	Human platelet lysate
ITS	Insulin–Transferrin–sodium Selenite supplement
PC	Platelet concentrates
PD	Population doubling’s
PDT	Population doubling time
SFM	Serum-free media
tPRP	Thrombin activated Platelet Rich Plasma
XFM	Xeno-free media

Introduction

Cell culture began in the 19th-century when a physiologist, Sydney Ringer, developed a solution capable of maintaining a beating frog heart outside of the body. He was able to maintain the tissue under living conditions by submerging in an isotonic salt solution composed of sodium chloride, potassium chloride, calcium chloride and sodium bicarbonate. (1) A buffering system, usually phenol red, was also added to the culture media in order to monitor a pH at physiological ranges. In the years 1907 to 1910, specific methodology for cell culture was established by Ross Granville Harrison. He was the first to be able to observe neural growth. In his experimentation, he placed fragments of embryonic tissues from frog medullary cords on cover slips of glass slides and sealed them with clotted lymph. He indeed generated the first moist chamber. (2) Apart from the nutrition, animal cells also need specific culture conditions; they are kept in incubators in a controlled humidified gas mixture of 5% CO_2 and 95% O_2 under a physiological temperature of 37° C. The CO_2 is essential to maintain the pH of media because without CO_2 the medium becomes alkaline and compromises cellular survival and proliferation.

Cell culture techniques made significant advances in the 1940s and 1950s to support research in virology. As viruses require the components of infected cells to reproduce themselves, large scale manufacturing of viral material in safe conditions necessitates co-culture with animal cells. For instance, the vaccine for Polio was produced with the aide of co-cultured fetal cells. Harry Eagle, an American pathologist, defined the minimal requirement in 1955 for the Hela cell line (isolated from a human uterus Carcinoma at Johns Hopkins Hospital) and mouse fibroblasts, laying down the fundamental principles of mammalian culture. Eagle proved that 27 elements were essential for the growth of mammalian culture, and his medium Eagle's minimal essential medium (Eagle's MEM) contains various amino acids, glucose, vitamins, isotonic salt solutions and an whole animal or human serum. (3) All of the elements were defined as the base medium without the serum component. Dulbecco formulated a modified version of the Eagle's MEM, Dulbecco's MEM (DMEM) around the same time period, with 4 times more vitamins as reported in Table 1. Historically cell culture has been done with the addition of serum to the culture medium to mimic the physiological condition of the body. The source of the serums can differ; it can be human serum, horse serum or cattle serum among the most common.
Serum provides the cells with nutrients, attachment factors, growth factors and hormones, binding and transport proteins, spreading factors, fatty acids, lipids and protease-inhibitors. All of these factors are essential for proliferation and differentiation of cells in culture systems.

Inorganic salts	MEM	DMEM
$CaCl_2$	200	200
KCl	400	400
$MgSO_4$	98	97.67
NaCl	6800	6400
$NaCO_3$	2200	3700
NaH_2PO_4	140	125
$Fe(NO_3)_3$	-	0.1
L-Amino acids		
Arginin	126	84
Cystine	31	63
Histidine	42	42
Isoleucine	52	105
Leucine	52	105
Lysine	73	146
Methionine	15	30
Phenylalanine	32	66
Threonine	48	95
Tryptophan	10	16
Thyrosine	52	104
Valine	46	94
Glutamine	-	584
Glycine	-	30
Serine	-	42
Vitamins		
Cholin	1	4
Folic Acid	1	4
Nicotinamide	1	4
Pantothenic acid	1	4
Pyridoxal	1	-
Thiamine	1	4
Riboflavine	0.1	0.4
Inositol	2	7.2
Miscellaneous		
D-Glucose	1000	4500
Phenol red	10	15

Table 1: Composition of Eagle's MEM and the Dulbecco's MEM Medium. Adapted from ref 1 and Yang and Xiong culture conditions and types of growth media for mammalian cells, 2012)

Fetal Bovine Serum

Serum is an essential requirement of mammalian cell culture since the beginning of the technique. Until now, Bovine serum is the most wildly used in cell culture, including, bovine calf serum, newborn calf serum and fetal bovine serum (FBS). Fetal bovine serum provides the highest quality because the fetus is not exposed to the outside environment and therefore presents low antibodies and complements. The main components of the serum are listed in Table 2.

Component	Mean concentration	Component	Mean concentration
Na^+	137mol/L	Alkaline phosphomonoesterase	225U/L
K^+	11 mol/L	Lactic dehydrogenase	860U/L
Cl^-	103 mol/L	Insulin	0.4μg/L
SeO_3^{2-}	26μg/L	Thyroid stimulator	1.2μg/L
Ca^{2+}	136mg/L	Folliclestimulating hormone	9.5μg/L
Fibonectin	35 mg/L	Bovine somatotropin	39μg/L
Urea acid	29 mg/L	Prolactin	17μg/L
Creatine	31mg/L	T_3	1.2μg/L
Hemoglobin	113 mg/L	Cholesterol	310μg/L
Bilirubin(total)	4 mg/L	Cortisone	0.5μg/L
Inorganic phosphorus	100mg/L	Testosterone	0.4μg/L
Glucose	1250mg/L	Progesterone	80μg/L
Urea	160mg/L	Prostaglandin E	6μg/L
Total protein	38g/L	Prostaglandin F	12μg/L
Albumin	23g/L	Vitamin A	90μg/L
α_2- macroglobulin	3g/L	Vitamin E	1 mg/L
Endotoxin	0.35μg/L	Fe^{2+},Zn^{2+}, Cu^{2+},Mn^{2+},Co^{2+}, Co^{3-},etc	μg/L to ng/L

Table 2: The main components of serum and their mean concentration. Zhanqiu Yang and Hai-Rong Xiong. Culture Conditions and Types of Growth Media for Mammalian Cells. "Biomedical Tissue Culture", book edited by Luca Ceccherini-Nelli and Barbara Matteoli, ISBN 978-953-51-0788-0.

For the clinical application of cell therapies and tissue engineering, reliable and safe cell culture methods are needed. Indeed the quality and safety of cell products are dependent on how they were obtained and how they are cultured over time.

An investigation on commercially available FBS has shown that a high degree of serum variability was present both within and between suppliers in all major categories investigated (see Figure 3). Therefore, caution should be employed in the interpretation of results from experiments using serum supplements without specific quantification of possible interfering or modulating factors. (4)

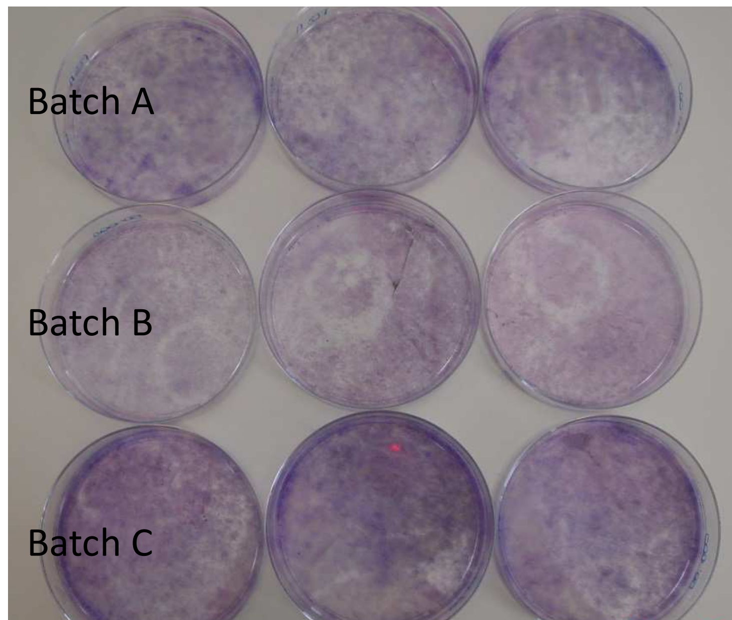

Figure 3: Variabiliy between 3 batches of serum (picture from UTR).Fibroblast cells were grown for 2 weeks with the same concentration of serum from 3 separate sources to evaluate overall cell growth. Cells were plated in triplicate, fixed in alcohol and stained with Giemsa to assess overall growth.

A recent study using proteomic analyses has shown that the composition of FBS varies over time in culture medium. The amount of several extracellular matrix and structural proteins, which are indicators of cell growth, decreased. (5) This vast batch-to-batch variability and the primitive control tools used by suppliers (such as testing osmolality, presence of endotoxins, and growth promoters) can be responsible for phenotypical differences in the cell culture and make it difficult to compare results from one laboratory to another. Secondary treatments of FBS, such as heat inactivation (30 minutes at 56°C with mixing to inactivate complement), can also induce metabolic and morphologic changes in cell cultures. For example, lymphoblasts show significant differences in their proteome accordingly if they were cultured in heat inactivated FBS or not. (6) FBS is also associated with a risk of transmitting various infectious agents, such as prion or virus. In fact, it has been reported that as much as 20-50% of commercial FBS is virus-positive. (7) Bovine proteins can also be internalized by the cultivated cells or can attach to their membrane which may lead to immunological reaction after cell transplantation. Specifically, a single injection of 100 million hMSCs grown under standard conditions (20% FCS) can carry between 7 to 30 mg of calf serum proteins, and rats who received several doses of HM-MSC showed strong immunoreactivity against bovine serum albumin.(8) Indeed, patients treated with lymphocytes cultured in FBS have developed anaphylactic or arthritis-like immune reactions(9); and cardiomyoplasty with skeletal myoblasts grown in an FBS enriched medium was shown to cause ventricular arhythmias and sudden death in patients. (10) However, the majority of clinical studies have been accomplished with MSCs expanded in media containing FBS, and were performed without the appearance of major side effects. Apart from the technical problems mentioned above there is an ethical issue about the collection of FBS as it is harvested from bovine fetuses taken from pregnant cows during slaughter. It is obtained through a cardiac puncture without any form of anesthesia. Therefore, it could be imagined

that fetuses are exposed to suffering during harvesting, so the current practice of fetal blood harvesting has been described problematic. (11)

FBS availability is also limited, as a byproduct of meat processing industry, many factors will impact the annual harvest, such as:

- Weather-induced cattle sell-offs (drought and harsh winters)
- Cattle retention (ample forage and government intervention in the agriculture market)
- Dairy cow buy-outs to reduce milk production
- Increasing milk and meat demand(12)

Two mega corporations control production and distribution of FBS worldwide. Associated with an increasing demand it could be imagined that high and volatile prices are expected for the next decade. Currently, one liter of clinical-grade Australian/ New Zealand serum costs over 1300 US dollars. (13)

Regulatory Framework

All tissues cultured within cell culture systems have to trace all elements that come into contact with the tissue from the beginning of harvest. Therefore, all nutrient media, growth factors and serum have to be of high clinical-grade quality if the cells are to be used in therapeutic applications.

There are different regulations world-wide but many have common ground.

USA

In the USA, human cells and tissues and cellular- and tissue-based products fall under the Code for Federal regulation 21, part 1270 and 1271. Examples include, but are not limited to, bone, ligament, skin, dura mater, heart valve, cornea, hematopoietic stem/progenitor cells derived from peripheral and cord blood, manipulated autologous chondrocytes, epithelial cells on a synthetic matrix, and semen or other reproductive tissue. Food and Drug Administration (FDA) requires firms to register and list their products. It also requires tissue establishments to evaluate donors, through screening and testing, to reduce the transmission of infectious diseases through tissue transplantation. Finally, it establishes current good tissue practices for cell and tissue-derived products. Development and clinical application will undergo specific regulation and is associated with the Center for biological evaluation and research, a subdivision of the FDA.

More information can be found under following this link:

http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=1271&showFR=1

Europe

The European tissue and cell Directive (EUTCD) is made up of three Directives, the parent Directive (2004/23/EC) which provides the framework legislation and two technical directives (2006/17/EC and 2006/86/EC), which provide the detailed requirements of the EUTCD. The directives set a benchmark for required standard when using tissues or cells for human application. This directive sets traceability requirements, notification of serious adverse reactions and events and certain technical requirements

for the coding, processing, preservation, storage and distribution of human tissues and cells. It applies to tissues and cells including haematopoietic peripheral blood, umbilical-cord (blood) and bone-marrow stem cells, reproductive cells (eggs, sperm), fetal tissues and cells and adult and embryonic stem cells. Blood and blood products are not concerned by this directive. Besides this directives the European Union (EU) has set quality standards known as Good Manufacturing Practice (GMP) 2001/83/EC to ensure that medicinal products are consistently produced and controlled against the quality standards appropriate to their intended use. Also, products made for research purpose are concerned. The products are controlled and validated by the European Medicines Agency. Compliance with these principles and guidelines is mandatory within the European Economic area. Switzerland as a third country (non member of the EU) has requested the EU to assess that its regulatory framework was applicable to substances exported and that the respective control and enforcement activities ensure an equivalent level of public health protection. This assessment was successfully performed in 2012.

More information can be found under the following link:

http://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1404288944041&uri=CELEX:52009DC0708

Switzerland

In Switzerland, tissue engineering and cell therapies are regulated within the Transplantation Law, together with associated directives. Manufacturing, distributing, marketing of cells therapies and tissue engineered products requires an authorization from Swissmedic and compliance to Good Manufacturing Practice and good documentation practice. Clinical trials have to be notified to Swissmedic and must be carried out in accordance with the guideline for Good Clinical Practices (GCP). Swissmedic must also be notified for any problem or accident. (14) Transplantation of cells and tissue from embryo or fetal origin are also submitted to authorization of the federal office for public health.

More information can be found under the following link:

https://www.swissmedic.ch/bewilligungen/00155/00242/00243/00245/00246/00249/index.html?lang=fr

Aim of the present study

Due to the previous exposed points there have been many attempts in replacing FBS with other products of different origins over the last years. In the following study we will try to have an overview of the existing options provided by the literature for the latest research with a special focus on adult human multipotent mesenchymal stem cells (hMSC) and multipotent adipose tissue-derived stem cells (ASC). Indeed, most of the research accomplished to date for the replacement of FBS was done on these two types of cells. However, many of the results can be extrapolated to other cells types and particularly primary cell cultures.

This work is based on a complete review of scientific articles. Two research engines were used: Pubmed and Sciencedirect. The principal key words used were the following «FBS; serum free; mesenchymal stem cells (MSC); human platelet lysate (HPL); serum-free chemically defined media”. The obtained list has then been completed with a manual research of supplementary references, focused on English articles dating within the last 10 years. The bibliography of retained articles was then further examined in order to extend the research. The free online media database was also consulted, permitting to see how commercial products were classified and the amount of information given by the Manufacturer. Explanations about the incoming challenges in tissue regeneration are also given for an immersion in the field and focus on the future to increase security for the patient.

An illustration of the adaptation procedure will be exposed at the end of this study. The Regenerative Therapy Unit of the CHUV is evaluating some of the different solutions to replace FBS in tissue engineered skin substitutes for severe burns, with both keratinocytes and fibroblast skin cells. We will show a first experimental part with the testing of 2 different lots of FBS for cell culture. Then, we will expose different results concerning the testing of PRP and human platelet lysate as potential replacement of FBS and finally we will report the evaluation of commercially available serum free mediums.

Serum Substitute Possibilities

In the last decade, because of the ever increasing potential of problems caused by FBS in cell culture and in the aim to apply new cGMP legislation there has been a steady interest of FBS ersatzes. Unfortunately, new alternative culture media often does not use a standardized terminology. Not knowing what the products contain, or what is exactly meant under which abbreviation can lead to confusion. It also appears that media manufacturers promote ambiguous and attractive terminologies in order to attract more customers, thus maintaining the actual current confusion. [17] Therefore, a brief summary of standardized classification is summarized in order to have a clear overview of existing products, the following classification is the one used by Kinzebach and Bieback. (expansion 15)

Serum-free media

Undefined culture media that is not supplemented with serum but may contain proteins fractions (eg. animal tissue or plants extracts) such as bovine/porcine pituitary extract, chicken embryo extracts etc. (15) Non-protein constituents may also originate from animal sources and the elimination of animal-derived protein elements is not enough to guaranty a xeno-free media. (16) For the reasons mentioned, the risk of transmitting xenogenic proteins and pathogens by serum-free media remains unchanged compared with FBS supplemented culture media.

Xenogenic-free media

Also called humanized culture medium. So far human plasma, serum, cord blood serum and platelet derivatives have been tested to replace FBS. Also, xenogenic-free media are undefined.

Animal-derived component free

Media containing no components of animal origin, they are not necessarily chemically defined (such as if they contain bacterial or yeast hydrolysates or plant extracts).

Chemically defined media

Do not contain proteins; hydrolysates or any other components of unknown composition. Hormones or growth factors added can be of either animal or plant origin or recombinant products. A chemically defined media using recombinant products represents the ultimate aims in cell culture, as all the components are well defined and characterized. This allows full standardization with the highest possible safety grade.

In order develop a serum-free medium for mammalian cell culture J. van der Valk *et al.* (2010) proposed a pyramidal approach with an incrementing specificity of media composition (Figure 4). He recommended to start a new formulation with 50:50 (v/v) mixture of DMEM (Dulbecco's Modified Eagle's Medium) and Ham's nutrient mixture F-12 plus an ITS supplement (insulin, transferring and

selenium). Coating of the culture vessel with extracellular matrix components, such as collagen or fibronectin, would also be possible in order to furnish ample cell attachment factors to the culture. Addition of specific hormones and growth factors, depending on the cell type sometimes could be added to the media and is often required. Therefore, adaptation of cells lines to the new culture conditions required a gradual lowering in serum concentration. To detect unwanted selection of cells capable to grow in serum-free media the author recommended close monitoring of cellular morphology and functioning during the adaptation process (Table 3). (17)

Indeed *"different isolation methods and culture conditions may lead to multiple MSC populations with slightly different biological and functional characteristics. Differences in culture medium or supplements, plating density, level of confluency at cell detachment may influence their proliferative capacity, expression of surface makers or differentiation capacity."* (18)

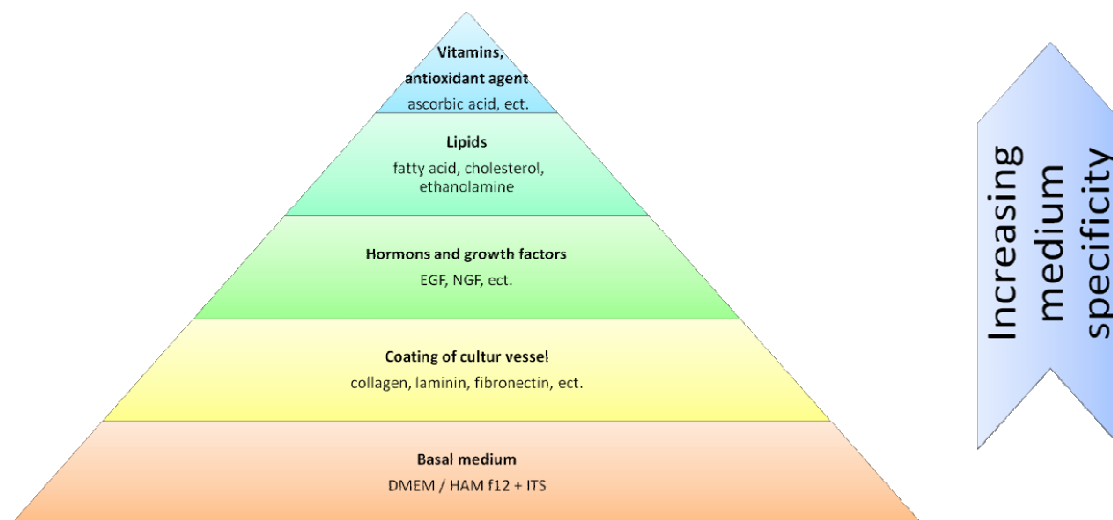

Figure 4: Pyramidal approach to build a serum-free medium. Inspired from van der Valk and Brunner; Optimization of chemically defined cell culture media – Replacing fetal bovine serum in mammalian in vitro methods.

Medium Constituent	Animal source	Non-animal substitute
Insulin K	Bovine/porcine pancreas	Bovine or human recombinant
Transferrin K	Bovine, porcine or human plasma fraction	Inorganic iron carriers/chelates
Serum protein fractions K F (e.g., albumin, fetuin, lipoproteins)	Bovine or other animal serum	Lipid delivery alternatives Plant-derived hydrolysates
Protein hydrolysates	Lactalbumin, peptones, casein	Plant-derived hydrolysates
Lipids/sterols	Ovine/human cholesterol	Plant-derived sterols Synthetic and plant-derived fatty acids
Growth and attachment factors K	Murine/bovine organ digests	Recombinant factors Collagen precursors
Amino acids (e.g., tyrosine,	Human hair; Avian feathers; Bovine	Recombinant, synthetic or
Coating agent	**Animal source**	**Non-animal substitute**
cyst(e)ine, hydroxyproline)	collagen; Bovine/porcine bone gelatin	plant-derived amino acids
Reactives	**Animal source**	**Non-animal substitute**
Surfactants (e.g., Tween 80)	Bovine tallow	Plant-derived polysorbate
Dissociating enzymes (e.g., trypsin) K F	Porcine pancreas	Plant-derived enzymes Microbial enzymes

K *stands for used in Keratinocyte culture*.
F *stands for used in Fibroblast culture.*

Table 3. Common cell culture media constituents and reagents derived from animal sources(16)

To ensure a total xeno-free culture, not only FBS but all the above mentioned media constituents need to be replaced by plant derived, recombinant or synthetic products. FBS products are by far not the only components that need to be replaced.

Human Blood Derivatives

All the humanized culture media which use blood derived products are submitted to the same national legislation as normal blood donation. As a consequence, safe use in clinical applications depends strongly under which conditions blood products are locally obtained. In Switzerland, three essential principles are applied in order to guaranty safety of blood products.

1) Selection of donors, according to the World Health Organization (WHO) donation has to be done on a volunteer basis, without reward or payment. Selection of donors is done via a questionnaire before every blood donation. The questionnaire has been elaborated with the aim of detecting high risk situations or behavior which could lead to a temporary or definitive exclusion from blood transfusion programs.

2) Laboratory testing of blood products. All blood products are routinely tested for the following pathogens after donation: AC anti-HIV1, AC anti-HIV2, AC anti-HCV, AgHBS, AC anti Treponema Pallidum.

3) Pathogen inactivation. Currently more than 25 blood transmissible pathogens exist and for the majority of them no detection methods exist. It can also occur that pathogen charge is too low to be detectable by conventional testing, such as in the first phase of an infection. This is why pathogen inactivation procedures make sense, allowing the inactivation of the broad spectra of potential unknown pathogens, whether they are fungal, bacteria, virus or parasites. Pathogen inactivation works in the following manner; first an intercalating agent is added to the blood product to link to the RNA or DNA of the pathogens. Further light activation of the intercalating agent provokes a block in transcription or a nucleic acid desaturation and these steps lead to final pathogen inactivation. (19)

The current method used at our hospital to block the reproduction of pathogens is the Intercept Blood System (Cerus Corporation, Concord CA, USA). This technique implies a photochemical component: Psoralen amotosalen and long range UVA irradiation. The amotosalen is added to the blood donation, then it binds DNA and RNA before being activated with pure UVA light (figure 5). Covalent bonds between pyrimidic base residues block further transcriptions of any pathogen.

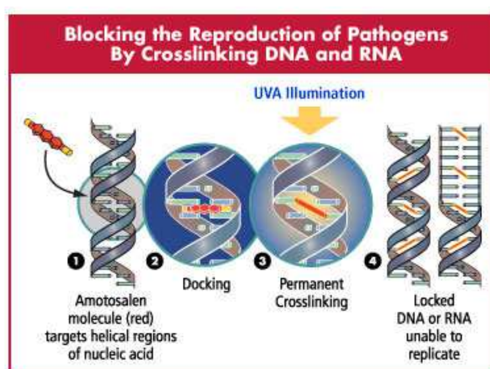

Figure 5: Intercept mode of action. *http://www.interceptbloodsystem.com/product-overview/amotosalen-mechanism-of-action*

The Intercept Blood System has been proven efficient in reducing the risk of transfusion transmitted disease and preventing the occurrence of Graft versus Host Disease (GvHD). Specific studies of platelet function also have shown that pathogen inactivation did not hinder the haemostatic capacities of treated platelets. The documented reduction of infection risk through this efficient method allowed the CHUV to expand the shelf-life of platelet concentrates from five to seven days. (20)

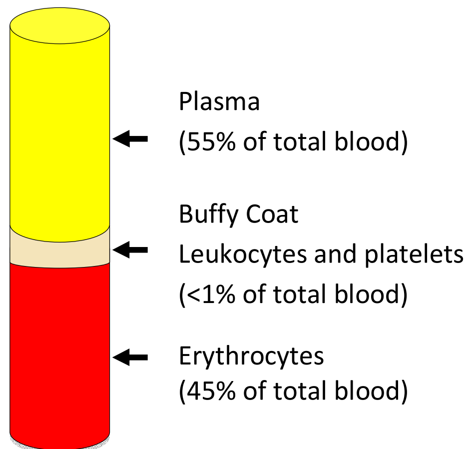

Figure 6: Blood components after whole blood donation and centrifugation with addition of an anticoagulant.

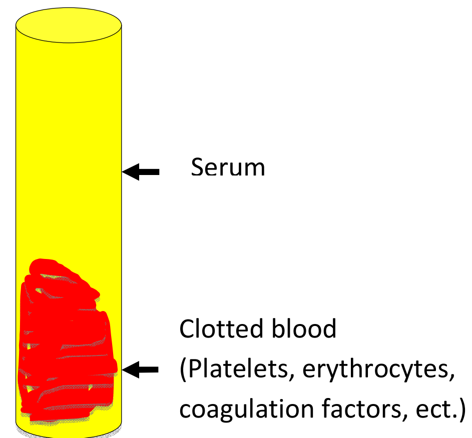

Figure 7: Blood components after clotting of a whole blood donation.

Human Platelet Lysate (HPL) and Platelet Rich Plasma (PRP)

Platelets are small circulating enucleated particles, essential for blood coagulation, wound healing and tissue repair. They are released in the blood stream through the apoptosis of progenitor cells called megakaryocytes, they circulate for 7-10 days before being replaced.

“Platelets play a critical role in normal hemostasis by forming the hemostatic plug that initially seals vascular defects, and by providing a surface that recruits and concentrate activated coagulation factors.” (21) After a vascular injury, platelets are activated through the contact with ECM constituents and undergo the following stages: 1) Platelets adhesion to ECM via von Willebrand factor; 2) Secretion via degranulation; 3) Platelet aggregation.

The platelet granules contain a cocktail of various substances such as fibrinogen, fibronectin, coagulations factors, platelet-derived growth factor (PDGF), TGF-β, ionized calcium, histamine, serotonin and epinephrine. (21) PDGF stimulates proliferation and chemotaxis of neutrophiles, macrophages, smooth muscle cells and fibroblasts to the wound site. (22) Due to the high content in platelets, and thus in growths factors, PRP is believed to have a therapeutic effect on wound healing. A systematic review about the efficiency and safety of autologous PRP in several clinical areas such as oral and maxillofacial applications, chronic skin ulcer, and wound healing after surgery concluded that tissue regeneration was significantly improved in chronic periodontitis and chronic skin ulcer. (23) Platelet

derived products are a suitable alternative for FBS. Indeed, many attempts in replacing FBS by platelet derived products in MSC cell culture were shown to be successful.

Human platelet lysate (HPL)

Human platelet lysate (HPL) can be obtained from sources such as single donor apheresis platelet concentrate or from whole blood donations using buffy coat-derived platelet concentrates. Alternatively, it can also be from plasma reduced concentrates with platelet additive or human albumin. (15) "When HPL is used for the expansion of cell therapeutics the donors have to fulfill the respective national regulation for blood donation." (24)

As there is not any established production method of HPL, each laboratory develops their own processing and end-product. This can possibly lead to differences in the proprieties of the HPL obtained and make it difficult in comparing outcomes. Therefore, we will attach a summary of production possibilities associated to the clinical result outcomes. Protocols for HPL production from buffy coats and apheresis derivate platelet concentrates are accomplished through freeze thaw cycle or chemical lysis which causes disruption of platelet membranes and release of growth factors. In a second step, centrifugation and filtering is needed to remove the platelet particles and membrane antigens; which bear the threat of alloimmunization against platelet antigens *in vivo*. (24) Indeed, various immune-mediated platelet disorders have been described such as thrombocytopenia, pos-transfusion purpura and platelet transfusion refractoriness.

A study comparing pooled whole blood derived PC to apheresis PC could not highlight any notable differences in the cytokine content or stimulation of MSC proliferation. The optimal HPL concentration for cell growth was determined to be approximately 10%. This study also showed the major synergic role played by PDGF, TGF-B1 and bFGF in MSC proliferation. If all factors were inhibited together, the HPL stimulating effect decreased by 75%. However, combination of recombinant PDGF, TGF-B1 and bFGF could enhance cell proliferation alone. Another interesting factor is that PC could be conserved up to 18 months without significant degradation or changes. (25)

Expired blood bank platelet concentrate can also be used in HPL production with the same efficiency as HPL from fresh platelet concentrates and thus without competing with the needs of patients for platelet transfusions. In fact, differentiation potential, cell surface antigen and immunomodulatory functions of BM-MSC remained unchanged in fresh and expired HPL supplemented cultures. (26) Moreover, the average generation time between population doubling was shorter in cultured cells and hence an increased proliferation potential of HPL when compared to FBS. Nevertheless, some differences could be detected. Osteogenic marker gene RUNX-2 expression was higher and cells showed morphological changes with more spindle shaped, elongated cell structure and showed denser cell-bodies. Also, the growth pattern of cells was modified to a mesh-like pattern with circular areas of no cell growth. (26)

Another study was done investigating BM-MSC culture. The cumulative number of cells until senescence was much higher in HPL containing media, but there was a decrease in adipogenic differentiation. Immunosuppressive activity was retained and microarray data confirmed the observation of rapid growth. Optimal number of thrombocytes needed for HPL- preparation was estimated at 1.5×10^9 /mL

with a proportion of 5% in cell culture but granular deposits in culture made the microscopic observation difficult. Pooling of PRP and titration of platelet concentration was shown to be a good way to normalize the amounts of growth factors and cytokines delivered by the end product. (27)

Schallmoser *et al.* produced 3 to 3.5L of pooled PRP from at least 50 donors, with a platelet concentration of 0.95×10^9 +/- 0.15×10^9 per mL. After, further manipulations were needed to obtain HPL, and BM-MSCs were then cultivated in an α-MEM media supplemented with 10% HPL and heparin. The proliferation rate of HPL-BM-MSCs was delayed until the sixth day, and further enhancement of proliferation resulted in a higher BM-MSC harvest. Morphological differences such as smaller more elongated cells could be noted, but these differences were not transposed into relevant immunophenotypical changes. Osteogenic and adipogenic differentiation potential remained unchanged. Tumorigenicity was tested by long term expansion, without the appearance of immortal cells. Also, subcutaneous injection of BM-MSCs in athymic nude mice with final animal dissection after a 90 days observation period was unable to find any tumor. (28) Further investigation of long term BM- and UCB-MSCs cultivation showed that phenotypical and functional characteristics of cells remained stable with HPL. Genetic studies were not able to find any chromosomal abnormalities, and telomere length decreased among the different passages with an absence of telomerase activity. (18)

When preparing HPL, pooling of PRP is recommended in order to diminish inter-individual differences in the amount of growth factors and cytokines delivered by each product lot. Cryopreservation of HPL also allows to re-test donors 3 month after blood donation for infectious markers. (29) Donor age can also have an impact on HPL quality. Indeed, umbilical cord PRP has been shown to have a higher concentration of mitogenic factors than adult PRP. Thrombocyte concentration will also be a determining factor in the amount of growth factors delivered by HPL. Comparison of different thrombocytes concentrations pointed out that a platelet concentration below $1,5 \times 10^9$ /mL reduced the pro-proliferative effect. Various pathogen reduction protocols have been established using photochemical treatment, without any difference in MSCs quality. (30)

Platelet Rich Plasma (PRP)

A recent study has concluded that thombine activated PRP was a suitable alternative to FBS in AT-MSC cell culture. Pooled buffy-coat platelet concentrates were activated alternatively through human thrombin, ADP/ epinephrine or TRAP-6 before being centrifuged and filtered. Out of these three products, thrombin activated PRP (tPRP) was determined as being the most efficient inducing agent with an expansion of 2.8 times higher than that of FBS. The authors point out that thrombin activation proceeded similar to physiological activation of platelets and thereby ensures the bioactivity of secreted growth factors. In addition, this method avoids the formation of platelet membrane aggregates, and the transfer of platelet membrane antigens. (31) ASCs maintained their tri-lineage differentiation capacity and immunophenotype. Colony forming units were similar to FBS, however with distinct cell densities and size. Nevertheless, results obtained with BM-MSC were more ambiguous, with a documented decrease in osteogenic differentiation. (30)

Comparison of platelet rich plasma concentration levels needed for ASCs and human dermal fibroblasts growth showed an optimal cell proliferation at 5% tPRP. Proliferation declined in a dose-dependent

manner in the presence of 10% or 20% tPRP. The level of PDGF-AB and TGF-β1 were 187 times and 81 times higher than those before platelet-rich plasma activation. (32) Activated platelet poor plasma (tPPP) was also capable of supporting an enhanced cell proliferation compared with inactivated PRP.

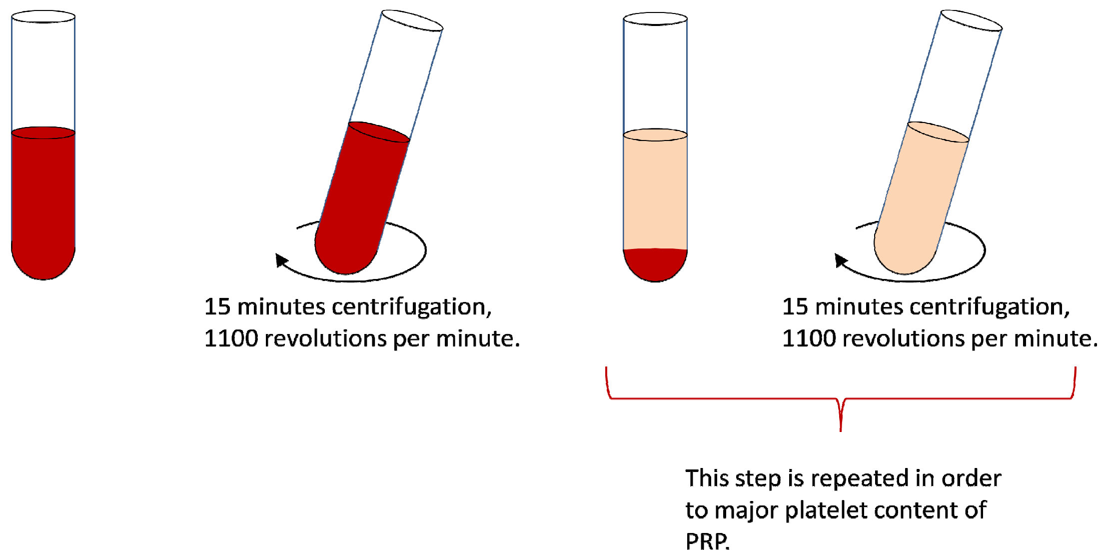

Figure 6: production of PRP in our laboratory based on the Standard Operating Procedure.

Human plasma

There are only few articles using plasma in MSC culture. As a matter of fact, natural clotted serum has been shown to be more effective than plasma in stimulating cell proliferation. This is likely because many factors promoting cell proliferation are released through platelet activation and clotting processes. (33)

A study using 10% autologous plasma was able to expand BM-MSC with a maintained tri -lineage differentiation capacity (adipogenic, chondrogenic, osteogenic). (34)

Human serum

Human allogenic serum

Two studies using commercial allogenic serum as a culture supplement have shown that fewer cells attached but were not able to form colonies. They were unable to expand and died after few cell divisions. Unfortunately, neither of the studies provided more information about the serum preparation and tests performed(8,35)and further interpretation of these results is therefore difficult.

On the other hand, a well documented study compared pooled HS (from at least five AB-blood-group typed donors, screened for the absence of HLA allo-antibodies and full fitting blood donor regulation) to FBS. AT-MSC cultured with AB-HS showed different morphology and growth patterns. They appeared smaller and spindle-shaped with fewer cytoplasmic processes than cells cultured with FBS, had a distinct

mesh-like growth pattern and a decreased adhesion potential, and they detached more rapidly from plastic after trypsination . The cumulative expansion rates were higher with AB-ALHS than with FBS. The differentiation capacity of AT-MSC was preserved and there was not any significant difference in surface markers expressed throughout long-term culture. “*Therefore AB-HS could be a widely available ersatz for FBS in AT-MSC cell culture, with only a few processing steps necessary. In addition, it shows the highest proliferative effect compared to FBS.”* (31)

Another study using ASC needed a 15% AL-HS concentration in order to reach the same proliferation rate than with 10% FBS. Statistical analysis of expressed surface markers showed no significant differences between both medium. However, oesteogenic differentiation capacity was decreased with AL-HS, chondrogenic differentiation remained unchanged and adipogenic differentiation was enhanced. Indeed, more oil droplets (lipid deposits) could be seen under light microscope and adipogenic markers were upregulated. Microarray gene expression analysis of 20,100 genes identified 1281 genes (6.4%) which where differently expressed. Significant changes were involved in cell cycle changes and the TGF-β signaling pathway (genes in this pathway control biological processes such as embryogenesis, morphogenesis of tissues like bone and cartilage, and angiogenesis). [27]

Human autologous serum

The limited availability of autologous blood in weakened patients is the principal obstacle to a generalization of human autologous serum in cell culture. “*Only 200mL of serum can be gained from a 500mL blood donation. i.e supplementing medium with 10% AS yields 2L expansion medium. what’s enough for a maximal 2-3 weeks expansion period, harvesting sufficient MSC for one to two clinical applications.”* (27)

A study analyzing characteristics of BM-HMC cultured in autologous serum pointed out that BM-HMC are morphologically and phenotypically equal to those cultured in FBS. On the other hand, proliferation rate was increased with a lowered differentiation rate, suggesting that FBS had induced cell lines further in the differentiation pathway and the changes in gene expression reflected this difference. Human autologous serum also tended to generate a more stable genomic background, with a delayed senescence process. [19, 20]

W. IM *et al.* compared the growth of ASCs in auto-HS to FBS and showed that ASCs had a greater expansion rate in auto-HS than FBS and that they expressed similar markers and conserved adipogenic differentiation capacity. However, chondrogenic and osteogenic differentiation capacities were not problematic. (36)

Chemically defined mediums

The serum-free media interactive online database

De Brunner *et al.* developed the serum-free media interactive online database (http://goodcellculture.com/). The database was an attempt to regroup all the commercially available serum-free media and medium supplements, divided in three categories: serum-free media, chemically-defined media and animal-derived component free media. The serum-free media were associated with different kind of cells through defined attributes such permitting a clear overview of existing products. Unfortunately the online database was closed in 2014.

Several issues have been mentioned about the use of commercial media. Their exact formulation is generally not furnished by the supplier and the products can be modified without informing the customers. Furthermore, supplements with the same name may differ in composition from one supplier to another. (17)

StemPro® MSC SFM XenoFree, StemPro® LipoMAX™, Invitrogen

Two serum-, xeno-free commercial products for the culture of AT-MSC were tested in the reported study. After a necessary pre-coating step of culture vessels with CELLstart (Invitrogen®), StemPro® MSC SFM XenoFree (SP XF) alone and the lipid-based enriched version: StemPro® LipoMAX™ (XF LM) were compared with 10% HS and 10% FBS supplemented culture. The results showed that xeno-free medium culture exhibited higher and prolonged proliferation rates while maintaining surface marker expression profiles and multipotentiality compared with FBS and HS cultures. Nonetheless, osteogenic differentiation capacity of XF LM AT-MSC was decreased, with spontaneous adipogenic differentiation occurring. Indeed, the AT-MSC where positive for Oil Red O staining and showed reduced ALP activity. According to the authors of the study, more rapid confluence rates may have caused spontaneous transdifferentiation. Also chondrogenic differentiation was distinctly established as the condensing pellets formed more rapidly than controls. SP XF cells were more homogenous and less scattered and had a larger proportion of cells dividing than HS and FBS treated samples. (37)

Serum replacement 1 and 2, Sigma-Aldricht

A study comparing 2 potential ersatzes for FBS developed by sigma Aldricht for ASCs culture medium showed drastic diminution of proliferation rates with severe alterations in differentiation capacities of ASCs. (38)

GlutaMAX™, Invitrogen

Experimentation substituting FBS in ASCs culture with GlutaMAX 1% demonstrated initially a decrease in cell growth compared with 10% allogenic human serum substituted cultures. Furthermore cells ceased to divide after 4 days and detached themselves from the cell culture flasks.

Experimental section

The Regenerative Therapy Unit laboratory is currently establishing new techniques for the treatment of burns victims, in particular they develop new products and test FBS substitutes in order to respect the Swissmedic standards and increase safety of final products.

FBS serum testing for primary cell culture

The Regenerative Therapy Unit has to regularly test new batches of fetal bovine serum for cell culture used in research and clinical purposes.

One FBS serum (Gibco_41F4344K) was tested for the capability to promote primary cell growth of 3 different cells lines: skin progenitor cells (FE002-sk2.R, p5.1), Tenocyte progenitor cells (FE002-TenR p 4.1, adult fibroblasts (NP/JATH-F p4). The growth capability was compared to the currently used serum (Sigma_011M3396). The culture medium was prepared as follows: DMEM (Gibco 41966-029) + 1% glutamine) and 10% of FBS. Each experiment was done in triplicate.

Cells were seeded at 3 different densities (1,000; 10,000 and 50,000 cells) into 10cm diameter Petri dishes. Medium was changed 2 times during the week. After 2 weeks of cultivation cells are fixed and stained with Giemsa to evaluate cellular confluence. Finally cell culture dishes were photographed (Figure 8).

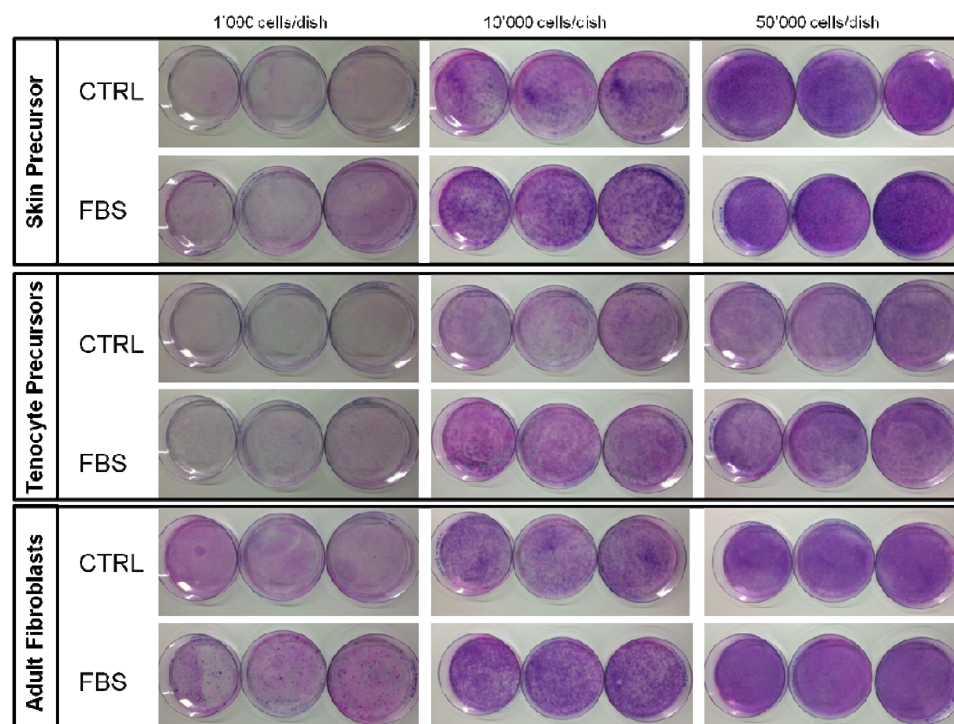

Figure 8: Comparison of new FBS lot to control after two weeks cultivation.

Cell culture images prove that this serum gives the closest result to control serum in this three cell lines. Therefore, it can now be used for research purposes on severely burned patients.

Human blood derived product production and testing

PRP and HPL

In this set of experiments, we aimed to evaluate the ability of PRP (platelet rich plasma) and HPL to promote cell migration by a scratch test assay. This assay is to represent a "wound model" *in vitro*. 250uL of PRP was obtained from the CPC (Centre de Production Cellulaire).

For the scratch assay, skin progenitor cells (FE002-sk2.R, p3) were seeded on 6 well plates at a density of $2x10^5$ cells per well in normal growth medium and cultured until they reached 90% confluence. Then, the medium was removed and cells were washed 1x with PBS. Once the PBS removed, marks were made on the bottom of the well with a marker (Figure 9), to help to find the location of the scratch for the photographic follow-up. Wells were washed again with 1x PBS to eliminate the residual scraped cells before adding 1mL of medium. Media were prepared as follows: Normal growth medium was composed of DMEM, 1% Glutamine and 10% FBS; PRP growth medium was made with DMEM, 5% PRP or 5% HPL and 1% Glutamine. The plates were then incubated at 37°C in 5% CO_2. Pictures were taken at 0, 24 and 48 hours after "wounding" with an Olympus X81. The most representative pictures are shown in Figures 10 and 11.

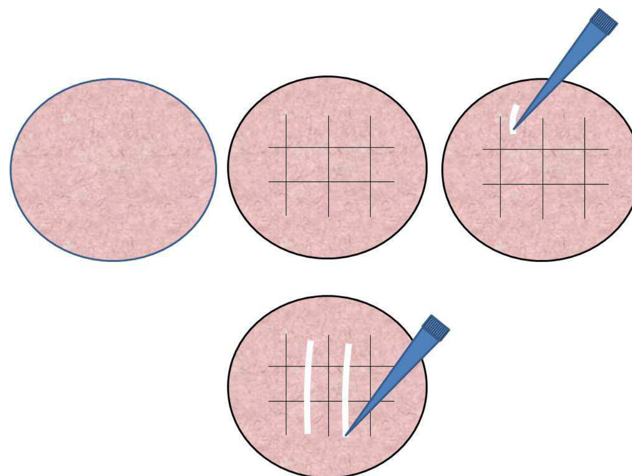

Figure 9: Schematic of the preparation of the cells for a scratch test assay.

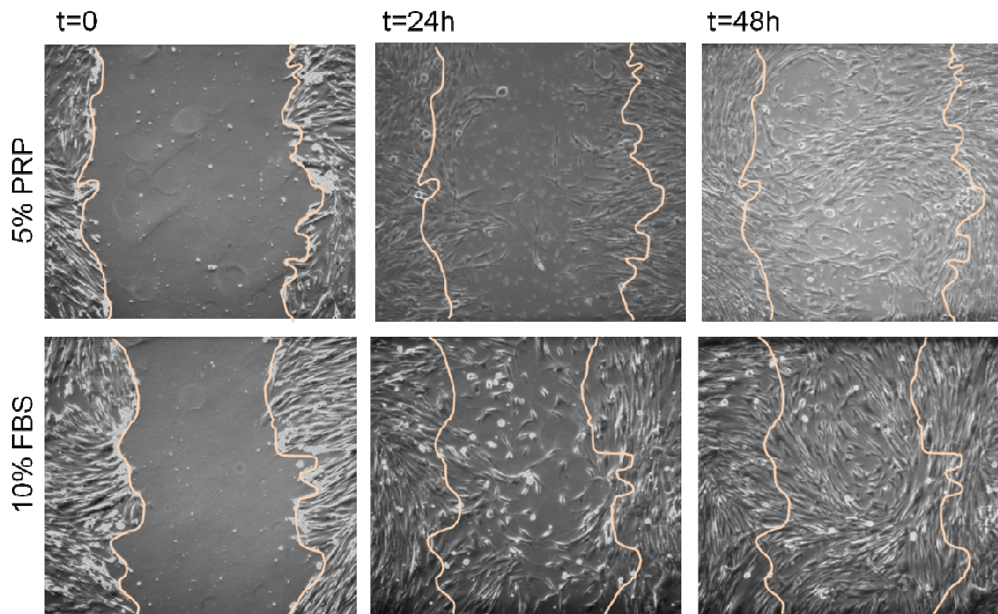

Figure 10: After 24 hrs post-wounding, a large amount of cells had already migrated into the scratch space in the 10% control medium condition. After 48 hours, the scratch zone was completely filled in both conditions. At the 5% PRP concentration, organized cell growth was rapid with non-altered cell morphology.

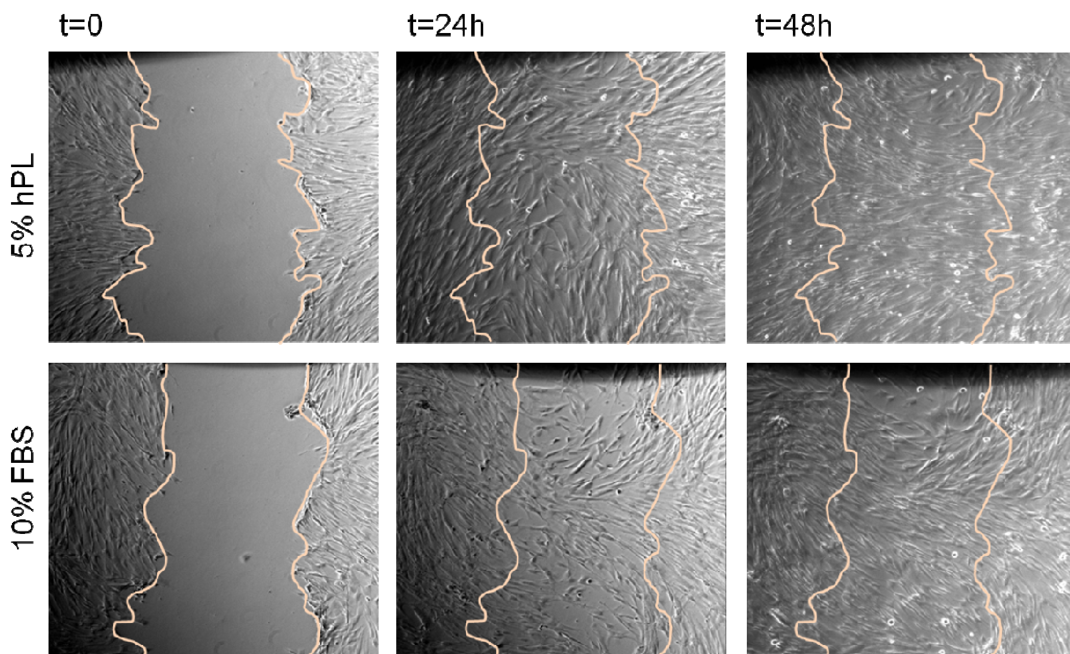

Figure 11: After 24 hrs of wounding a large amount of cells had already migrated into the scratch space in the 5% hPL. After 48 hours, the scratch zone was completely filled in both conditions. At the 5% hPL concentration, Cell growth was more rapid than in the control 10% FBS medium.

Example of commercial mediums

In this study, the UTR laboratory technician evaluated two serum-free media from a US company. She seeded 1,500 fibroblasts per well in a 96 well culture dish. The wells were filled with 100 uL of the different mediums.

Cell activity was measured after 0, 4, 7, 14, 18 and 21 days in culture using a Cell Titer 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA). The reagent of this assay contains a tetrazolium compound, MTS which is reduced by metabolically active cells into a formazan dye is optimally measurable at an absorbance wavelength of 490 nm. At each measuring time point, the old media was replaced with fresh media and MTS reagent. Samples were then incubated for 45 minutes at 37°C and the absorbance measured at 492 nm using an Absorbance Microplate Reader (Tecan) (Figure 12).

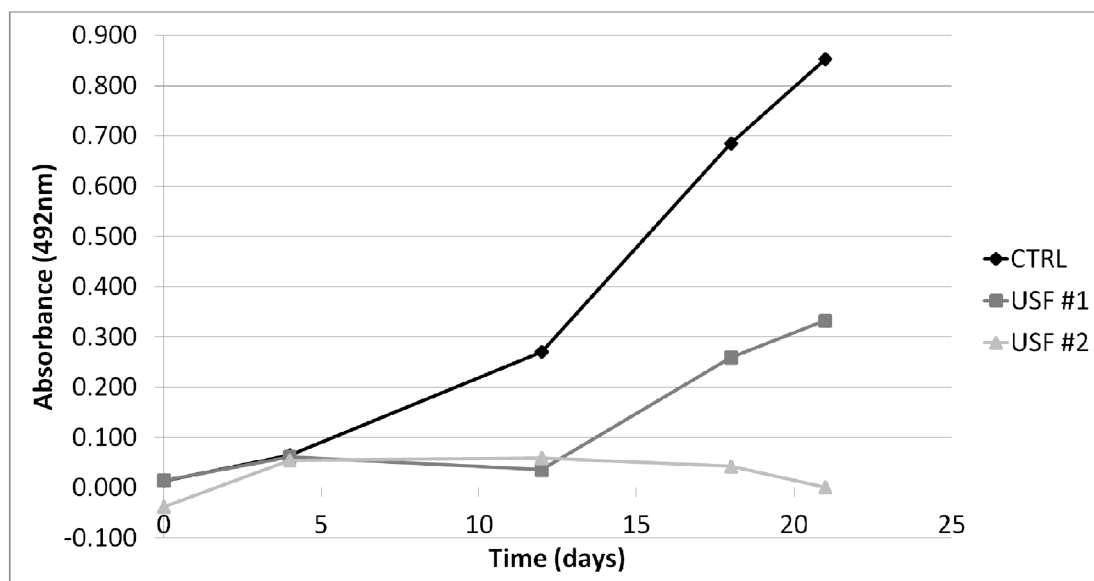

Figure 12: One of the US serum-free medias can be considered for cell culture, even if the cellular proliferation is notably reduced compared to the control medium (DMEM, 10% FBS, 1% glutamine).

Discussion

Obviously, FBS will be replaced by one or more products in the next decade. The optimal product should not contain any animal or human components, i.e. be completely defined, show at least similar proliferation rates to FBS, not alter the cell function and differentiation capacities and in addition be available at a competitive price. From where the leading successor will come is still unknown but it will probably be produced by some biotechnological enterprise. Indeed, the current legislation pushes the laboratories to promote industrial products to replace homemade preparations to be compliant with GMP manufacturing. As all the production steps of a clinical compatible cell therapy have to be validated by national control organs and proven GMP compatible, it is much easier to buy a industrial product that has already been granted serum-free and GMP compatible than to go through all the validation processes with a homemade preparation.

However, we will expose the following advantages and disadvantages of currently existing ersatzes

Industrial ersatzes

Due to the complexity of FBS, the current substitutes developed by industry are rather destined to specific cells types than universally adaptable. Therefore, only the most used cell types will have their serum-free media while neglecting the exceptions. Furthermore, FBS substitutes are not sold anymore as separate components but are rather part of so called “all-in-one” media. On one hand, it can bring cell culture to a higher level of standardization, but this can drastically dismiss the possibility of making personalized mixing.

Another issue with manufactured products is that their formulation can be modified at any time and without necessarily informing their customers. The lack of a standardized nomenclature used by all manufactures also makes it difficult to know what the products really contain. The used terms are often vague and unclear (for instance serum-free). Most products that are certified as serum-free products still contain animal- or human-derived products, therefore it should be carefully checked if the product composition is associated with any advantages in terms of biological safety compared to the actual medium used. Importantly, it is necessary to test the composition and watch for morphological and functional differences in specific cultivated cells.

It is also hard to know which media are currently on the market and eligible for your cell culture. The serum-free media online data base was a good attempt of centralizing all the existing serum-free commercial media; however it did not permit to have an overview of the efficiency of registered media and has been recently closed. The closure of the database contributes to favor the visibility of major players at the detriment of less known brands. A new website working on a cooperative basis would improve the information sharing. For instance, by allowing the different research laboratories to comment and rank the medium used and also to add links to published research done with cells cultured with a specific media. At the moment, no database registering serum-free culture media exists.

Lately, some interesting substitutes for ASC culture have appeared. Two commercial media have shown already comparable or enhanced proliferation rates than with FBS which was associated without significant alteration of cells characteristics: StemPro® MSC SFM Xeno Free and Knockout™ SR.

Knockout™ SR is guaranteed serum-free. StemPro® MSC SFM Xeno Free is guaranteed xeno-free, without further information about their content and what the company understands under the xeno-free term. Due to their probable content of human derived components, the safety level of those products is not necessarily higher than a homemade xeno-free media supplemented with human blood derived products. However, it seems an important milestone on the way to animal free and chemically defined media.

Human substitutes

Many problems have been pointed out resulting from the usage of FBS in cell culture also apply for Blood derived products. Indeed, allogenic proteins can also be internalized in cultivated cells and possibly lead to immunological reaction after transplantation. Furthermore, the Infection risk also remains; indeed human derivate products can also bare infection risk. However, intervention such as donor selections and pathogen inactivation permits to diminish to acceptable levels and cGMP compatible production. Currently in Switzerland, blood products are routinely tested for AC anti-HIV1, AC anti-HIV2, AC anti-HCV, AgHBS, AC anti Treponema Pallidum followed with pathogens inactivation with the Intercept Blood System. Further testing might enhance the security of blood products, however at higher costs. Similar to FBS, blood derived products also present a high batch-to-batch variability, which can be reduced by pooling and titrating of different lots.

There is also an ethical issue about the use blood-derivate products in cell therapy. In most countries blood derived products are often a limited resource, used in clinical therapies of many other diseases: such as acute blood losses, coagulation troubles, etc. Due to population aging and an increasing demand for blood donations, the availability of blood-derived products for research purposes will furthermore diminish. In Switzerland, an annual increase of around 10% in the amount of platelets used has been reported on average since 2000. (20) Therefore, it seems improbable that enough blood-derived products will once be available for cell culture at an industrial scale, without a drastic increase of blood donations.

Using expired platelet concentrates can be a way to avoid direct competition for blood-derived products. Actually, validity for HPL issued from expired platelet concentrates has been assessed up to seven days. (26) However, hospitals also tend to expand the validity period of Platelet concentrates for transfusion, diminishing the pool of available platelets for research. Platelet derivatives seem to be suitable alternatives to FBS. Indeed cells cultivated in HPL and thrombin activated PRP substituted culture medium, showed higher expansion rates without significant modification of cell functions and characteristics compares to cells grown FBS substituted cell cultures.

It seems likely that blood-derived products will be an essential asset in the bridging from animal-derived products to full xeno- and human-free products. How long the transition will take to have a fully xeno- and human-free product is still unknown.

The empire strikes back

Aware of the threat to their business caused by biosafety and ethical issues, 15 FBS producers created a lobbying structure defending their interest; the International Serum Industry Association (ISIA). The aim of this association is to standardize serum quality and integrity assessment tests and simplify their documentation to establish an appropriate Certificate of Analysis. At the moment, minimum testing standards already exist, however ranges of acceptance of the results are broad wich can differ from producer to producer. (39)

Furthermore, the ISIA also propose specific definitions for the origin of slaughterhouse-derived materials and donor origin materials. Unfortunately, the ethical issue about possible suffering of fetal bovine during harvesting seems to not be a priority. Indeed, no document delivered by the ISIA deals with the subject.

Conclusion

Ultimately, a chemically-defined media, produced on a large scale in well protocoled and standardized steps seems the more appropriate than FBS ersatz. The standardization process would virtually eliminate the batch-to-batch variation in the growth factor content. Infection risk would be reduced on the bacterial contamination of bioreactors needed in the production of the various components. Cell culture would not use any animal or human derived components anymore. In the meantime, use of blood-derived products seems a sustainable alternative. To avoid competition with use in routine hospital practice, the use of expired platelets in order to produce GMPc compatible HPL and thrombin activated PRP, would be recommended. Expired platelets also present the lowest possible cost, taking into the fact that they are thrown away if not used. For ASCs StemPro® MSC SFM Xeno Free Supplement, StemPro® with LipoMAXtm Defined Xenofree Lipid Supplement can already be used as an alternative to FBS, with a clear improvement of certain cell type proliferation.

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