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The Use of Fetal Bovine Serum in Cellular Products for Clinical Applications: Commentary

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Abstract: It was Eagle who first showed that culture media supplemented with animal sera was able to successfully maintain mammalian cells in culture, long term. Due to its widespread avaibility and easy storage it's become common practice to supplement cell culture media with animal sera. FBS (Fetal Bovine Serum) is sourced from donor cattle, and is the portion of plasma remaining after the natural coagulation of the blood. FBS is rich in growth stimulating factors and hormones that are synergistic in their stimulatory activities. At the same time, it's been demonstrated to have low levels of antibodies and gamma-globulin compared to other animal sera, which has led to the wide use of FBS as the main medium supplement in the production of research products and cellular therapies for clinical applications. Serum production takes place under very tightly controlled conditions and rigorous control of every step has to be maintained for clinical application. The use of FBS in the manufacture of cellular therapy products for clinical applications has been criticized in the last few years. However, a number of publications indicate that the transition to xeno-free protocols seems to be slow. The majority, i.e. over 80%, of regulatory submissions to the FDA describe the use of FBS during manufacturing of cellular therapeutics for clinical use.

Introduction

Cell culture techniques have remained largely unchanged since the work of Henry Eagle in 1955. In his 1955 paper Eagle summarized the works that lead him to chemically identify the specific nutritional requirements that supported growth of two mammalian cell lines – one a mouse fibroblast the other a human carcinoma – and showed that the omission of a single essential nutrient from the mix resulted in the early death of the cultures.

Prior to Eagle's pioneering studies, the growth of mammalian cells *in vitro* was supported by explants of tissue pieces, a biological matrix such as a plasma or fibrinogen clot where the growing cell attached, and a liquid media composed of human placental serum or adult serum, chicken embryonic extract and a balanced salt solution. Usually, this type of cocktail could support cell growth for a variable length of time, after which the culture died.

Eagle's detailed investigation identified a Minimum Essential Medium (MEM), which contained a mixture of 27 components: 13 amino acids, 7 vitamins, glucose, 6 salts, cofactors, and carbohydrates, all of which were necessary to support cell growth[1]. Omission of any of the 27 components resulted in cytopathic effects that could be initially reversed by replenishing the missing component. At the same time, if the component was not replaced, its absence caused the cells to degenerate and die in culture. The medium Eagle worked with was supplemented with dialyzed horse serum, required to support growth of mammalian cells inferred to have similar requirements for in vitro growth[1].

Most basal supplemented cell media cannot support the growth of mammalian cells by themselves. Eukaryotic cells, for their *in vitro* proliferation, have been found to require a poorly defined supplement of biological fluids and extracts. Both the former and the latter can be found in animal and/or human sera. Due to its widespread availability and ease of storage, it's become common practice to supplement cell culture media with animal sera. Serum additives in mammalian cell cultures are typically provided by fetal bovine serum

(FBS), capable of supporting the growth of a variety of cell types[2]. FBS, with its rich content of growth stimulating factors and hormones that are synergistic in their stimulatory activities, and low levels of antibodies and gamma-globulin compared to other animal sera, have led to its wide use as the main medium supplement in the production of research products and cellular therapies for clinical applications[3].

FBS is the portion of plasma remaining after the natural coagulation of the blood; during this process plasma protein fibrinogen is converted to fibrin, leaving behind a clot normally removed by centrifugation. While the procedure for making may seem to be straightforward, it takes place under very tightly controlled conditions. Serum production has been carefully developed over the years; it uses sophisticated facilities and equipment, and it is accompanied by extensive in-process and final product testing. Process control and product testing are particularly stringent for FBS intended for use in the production of biologics.

Despite the fact that FBS production has slowly developed into a highly standardized process with the final product tested for a wide variety of adventitious viruses to assure its safety, critical issues remain to be resolved. First, if and when various cellular therapy products that utilize FBS in their respective manufacturing processes become commercialized, issues of supply and demand need to be addressed; it's highly unlikely that current FBS production would satisfy the demand. Second, the use of xenogeneic proteins in the manufacture of cellular products designated for clinical use is still associated with potential risk of contamination by non-human pathogens such as prions, viruses and mycoplasmas, as well as initiation of the unwanted immune response to xenogeneic proteins in the immediate post-transplant period. A number of alternatives to the FBS – such as human serum albumin (HSA), human serum (HS), autologous serum, and serum-free media – have been investigated and demonstrated to be equally or more effective for *ex-vivo* cell culture and expansion of a number of cellular therapy products[4]. However, despite considerable progress in the development of cell culture techniques, including the development of the serum and protein-free media that are now routinely used to support mammalian cell growth, FBS supplemented media are still commonly used in clinical applications.

Fetal bovine serum production

Bovine serum is a by-product of the meat industry. Bovine blood can be collected at the time of slaughter, from adult cattle, calves, very young calves or from bovine fetuses. It can be also obtained from what are called "donor animals", which are raised to give blood more than once. Fetal blood is available because a small percentage of the cows designated for slaughter for meat production are found to be pregnant. Irrespective of whether blood is collected at slaughter or from donors, the age of the animal is an important consideration as it impacts the characteristics of the resulting serum.

The bovine fetuses from which blood is drawn for FBS production are obtained from pregnant cows which are sent to slaughter for reasons such as crippling lameness or when slaughtering herds of extensively kept beef cattle. Most often, bovine fetal blood is harvested by cardiac puncture via a closed system of collection. The advantage of this method is that it minimizes the danger of serum contamination with micro-organisms from the fetus itself and its environment[5]. The disadvantages are several. First, fetal cardiac puncture is performed without anesthesia, which has become a hotly debated ethical consideration in the last few years. It's been postulated that if the fetus is conscious during this procedure, it undoubtfully experiences a great deal of pain. Such inhumane treatment of animals is unacceptable to those concerned with animal welfare. Second, cardiac puncture is a procedure that requires specifically trained staff, which represents an investment on the manufacturer's side, with the cost passed on to the consumer. Fetuses are normally at least 3 months old, otherwise the heart is too small for the puncture. The cardiac puncture is performed by inserting a needle between the ribs directly into the heart of the un-anaesthetized fetus, with the blood extracted under vacuum into a sterile blood collection bag via a tube. In the absence of vacuum pump fetal blood may be obtained by means of gravity or massage. The volume if serum obtained depends on the size and, therefore, the age of the fetus. On the average, approximately 50% of the serum remains after clotting. A bovine fetus of 3 months yields approximately 150 ml of FBS, at 6 months 350 ml can be obtained, while at 9 months (considered to be term) approximately 550 ml of serum is produced. The global production of the FBS is estimated at 500,000 liters, which means that approximately 1,000,000 fetuses are required to meet the demand, which is not always possible[4,5].

Due to the fact that final FBS product can not undergo terminal heat sterilization as it would lead to the destruction of a number of biologically active constituents, the process utilized to produce the final product is tightly controlled and carefully documented. Aseptically collected fetal blood is refrigerated immediately after collection until coagulation is complete. The blood is then centrifuged to separate the serum from the blood clot, after which serum is removed. The resultant serum is kept frozen in carefully identified clean, hygienic containers until it is thawed immediately prior to further processing, as serum - like many materials of biological origin - deteriorates over time if not properly stored. In order to retain its properties, the resulting serum is stored and transported frozen. Great care is taken to ensure that proper storage and transport conditions are maintained at all times.

A pre-defined quantity of serum is processed and tested at one time to form what is called a "batch" or "lot" of processed product. Lots are triple 0.1 micron filtered, a process that has been validated to remove bacteria, fungi and mycoplasma. Serum is aseptically dispensed into gamma irradiated sterile bottles in a HEPA filtered Class 100 cleanroom. Immediately following the fill, FBS is frozen at (–10°C) - (–80°C), to preserve its quality. Product stability is usually guaranteed for 5 years from the date of manufacture, if the product is stored according to the manufacturer's instructions. Due to the fact that viruses are not removed by micro filtration, FBS is gamma-irradiated, the process which inactivates viruses. Gamma-irradiation is performed using a validated process, to assure that the correct dose of gamma rays is delivered on a consistent basis. As part of "batch" release, each FBS batch is tested for adventitious agents, to assure it's free from potentially contaminating viral organisms that include, but are not limited to Bovine Viral Diarrhea (BVD), Infectious Bovine Rhinotracheitis (IBR), Parainfluena Type 3 (PI3). In addition, each lot of FBS is tested for sterility, i.e. absence of aerobic and anaerobic microorganisms, mycoplasma content, endotoxin levels, hemoglobin and IgG concentration, as well as other moieties, as outlined in 21 CFR Part 210 & 211. Cell culture assay is usually performed on each lot to assess potency. Results of all tests must attest to the fact that each FBS lot meets or exceeds stringent previously established quality standards. At the same time, FBS remains chemically undefined as to the type and concentration of growth factors and hormones it contains. Hence, supplementation of basic growth media with FBS introduces a great deal of between-batch variability, which leads to inconsistent cell culture results. This variability is eliminated only subsequently to screening each FBS batch to assure its consistency and reproducibility in terms of cell growth characteristics[4].

The geographical origin of the material is also very important. Bovine blood is collected from herds grown in countries with a known, well-monitored, documented and acceptable animal health status, and which the US Department of Agriculture (USDA) has approved for import. This decision is based on the information published by the World Organization for animal health, which establishes the animal health status of countries or regions. Among the countries USDA recognizes as free of exotic diseases such as Foot and Mouth Disease (FMD), Rinderpest, Swine Vesicular Disease (SVD) and Bovine Spongiform Encephalopathy (BSE) are North America, Central and South America, Australia and New Zealand. In addition, countries recognized by the USDA must also have previously established infrastructure for maintaining authority-licensed slaughter houses, dedicated to safe processing of healthy animals, Hence, even in USDA recognized countries, animal herds must be inspected both before and after slaughter, by a qualified authority recognized as an expert. The fact that a given herd is deemed safe is documented to ensure that established procedures are followed and appropriate level of quality is attained. Each lot of FBS comes with a Certificate of Analysis (OA) and a Certificate of Origin (COO). The Certificate of Origin ensures that each lot is traceable back to the USDA abattoir in the country of origin.

Records are normally maintained for a defined period of time, in order to ensure that heard selection, each processing step, and the results of available testing performed could be linked directly to each FBS batch produced and received by the end user. The "traceability" of each material utilized to produce each batch of serum, from the origin of the heard to the final FBS product is paramount, especially when FBS is used in the manufacture of investigational biologic products designated for clinical use.

Use of FBS in the Manufacture of Human Pharmaceuticals and Biologics

Although the use of FBS in the manufacturing of cellular therapy products has been criticized in the last several years, a number of publications indicate that the transition to xeno-free protocols seems to be slow. The majority, i.e. over 80%, of regulatory

submissions to the FDA describe the use of FBS during manufacturing[6]. Although these statistics were used to describe mesenchymal stem cells (MSC)-based clinical trials, similar statistics are applicable across a wide range of cellular therapy products. The range of FBS concentration in media ranges from approximately 2% to 20%, with 10% FBS most commonly used. As mentioned elsewhere in this commentary, different concentrations of FBS result in different amounts of hormones and growth factors present in FBS. Therefore, most biologics manufacturers qualify FBS batches in order to achieve lot-to-lot comparability[6].

Processed FBS has a wide here range of applications. Perhaps one of the most important is the field of pharmaceuticals and biologics, where FBS is used in cell culture research, for product R&D purposes, and in manufacture of biologics, i.e. cellular products for clinical applications as they are regulated by the US Food and Drug Administration (FDA). Many of the cellular therapy products are at the cutting edge of drug development[5,6].

Natural clot serum is more effective than plasma in stimulating cell proliferation. This appears to be due to the release of certain polypeptides from activated platelets during the clotting process. A number of growth factors and essential minerals have been identified as the ones that drive specific gene expression, initiate and control the cell cycle and cell division, and program specific cell differentiation. As already stated elsewhere in this commentary, major role of serum in culture media is the delivery of hormonal factors which have several functions. The first one is to stimulate cell growth and proliferation, and promote cell differentiation and attachment. The second one is to assist in the transport of proteins carrying hormones, minerals, trace elements and lipids, stabilizing and detoxifying factors, all of which are needed to maintain optimal pH, or to directly or indirectly inhibit proteases such as a-antitrypsin or a2-macroglobulin and other toxic molecules[5].

For clinical applications, rigorous control of every step in the production of FBS has to be maintained. The closely controlled and validated production process, from collection to final product release, ensures minimal risk of contamination with adventitious agents, freedom from bacterial and fungal elements, low endotoxin content, consistent amount of IgG and hemoglobin, as well as other FBS constituents. This translates into lot-to-lot consistency and superior performance.

In the US and Europe, a number of regulatory bodies are involved in setting standards and defining requirements impacting the use of bovine serum in the manufacture of cellular therapy products destined for clinical use. In the US bovine serum products are regulated by the FDA and must follow the practices and procedures outlined in the United States Pharmacopeia (USP) and US Department of Agriculture (USDA). Both USDA and USP have developed regulations that address conditions and methods of slaughter, storage, manipulation, processing, treatment, testing, storage and transport of the bovine serum-derived products, to minimize the risk of contamination with adventitious organisms. Established regulations ensure that bovine serum is made from blood collected in countries with a known, well-monitored and documented, and acceptable animal health status. In Europe, the agencies involved in the regulation of the bovine serum production are European Commission, the European Directorate for the Quality of Medical and Healthcare (EDQM), and the European Medical Agency (EMA). The responsibilities of EMA are to produce scientific guidelines that cover development, manufacture and control of medicinal products[7]. By mandating that bovine serum is extensively tested for a variety of adventitious agents, both the US and European regulatory agencies ensure that processed bovine serum utilized in the manufacture of pharmaceuticals or cellular products designated for clinical use, meet the requirements established in these documents. The testing requirements established by EMA and USDA share common ground, with the major emphasis given to quality control, quality assurance, origin of country and traceability of the product, as well as testing of the final product, as outlined in the relevant published regulatory documents.

More than minimally manipulated investigational cellular therapy products for clinical applications are regulated as biologics, and must be manufactured in accordance with current Good Manufacturing Practices (cGMPs), described in detail in 21 CFR Part 210 & 211. These codified federal regulations – put forth by the US FDA – is a complex set of rules and requirements for any pharmaceutical manufacturing process. In order to ensure that the final product is safe, pure, potent and efficacious, GMPs impose minimal requirements for the facilities, equipment, and product and process control. The latter includes close control, standardization and testing of the raw materi-

als utilized during any manufacturing process, with close attention devoted to the FBS processed under strictly controlled conditions.

Serum changes the physiochemical properties of the cell culture media, including viscosity, osmolality, buffering capacity and diffusion rates. It helps to protect the cells from mechanical damage that may occur in stirred cultures or while use a cell scraper. It can be used in a wide variety of cell cultures despite the varying growth requirements by different types cells.

Recent studies have demonstrated the potential of bone marrow (BM) derived MSCs (BM-MSCs) as viable regenerative therapies for repair of various organs, including the heart. Since the first clinical trial of BMC injection in 1995, over 2000 patients have been treated with allogeneic or autologous MSCs for the treatment of various diseases including graft-versus-host disease, hematologic malignancies, cardiovascular disease, neurogenic disease, autoimmune diseases, refractory wounds, bone/cartilage defects, and as immunomodulation therapy for organ transplantation. A number of already completed and currently ongoing clinical trials have clearly demonstrated feasibility and safety of this therapeutic approach[8,9]. However, although some degree of efficacy has been achieved in a small number of clinical trials, demonstration of efficacy of BM-derived MSC therapy across a number of disease states remains an elusive goal[10].

To address this issue, Ikebe and Suzuki of the University of London looked at the methodology for the isolation, and culture and expansion of BM-derived MSCs utilized in over 200 already completed and currently ongoing clinical trials of MSC-based therapy listed on the website of the US National Institute of Health (NIH, http://www.ClinicalTrial.gov). They selected 47 reports of clinical trials that utilized this type of therapeutic approach, the results of which were published between 2007 and 2013. Sixty six (66%) of the clinical trials utilized autologous MSCs, with the remaining 34% using allogeneic cells[7]. The authors looked at the number of factors that impact the final product, which included but were not limited to the staring population for the MSC culture, i.e. whole BM vs. mononuclear cells (MNCs); seeding density; cell culture vessels, media and supplements utilized during culture; and duration of culture. Data collected in the course of this study demonstrated that FBS has been used more frequently (10% FBS in 73% of clinical trials and 15-20% FBS in 5% of the studies) as a growth factor and hormone supplement during culture[7]. The authors noted that despite a highly regulated and controlled environment in which FBS is now produced, shows great variation in terms of growth factor and hormone activity. Hence, great amounts of batch-tested for cell growth characteristics FBS needs to be reserved if and when MSC-based therapies become commercialized.

Successful use of FBS during culture and expansion of MSCs as well as other stem cell types have been reported over the years. Karussis et al evaluated the feasibility, safety and immunological effects of intrathecal and intravenous administration of autologous MSCs in patients with multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS), in a phase 1/2 open-safety clinical trial. MSCs were cultured for 40 to 60 days in a medium supplemented with 10% FBS[11]. With no major adverse effects of MSC infusion reported, the authors reported an increase in CD4+CD25+ T regulatory cell population, a decrease in the proliferate responses of lymphocytes, and a decreased proportion of activated dendritic cells. Le Blanc et al reported the results of patients with steroid-resistant, severe, acute graft-versus-host disease (GVHD) – a life threatening complication after allogeneic transplantation of hematopoietic stem cells (HSCs) – treated with *ex-vivo* expanded bone marrow (BM)-derived MSCs, in a multicenter, phase II clinical study[12]. Clinical grades MSCs were generated under GMP conditions. Specifically, BM mononuclear cells (MNCs) were separated by density gradient centrifugation and re-suspended in a cell culture medium supplemented with 10% FBS. The study demonstrated that MSC expansion protocol was safe, and MSC therapy was a safe and effective treatment for patients with severe, acute GVHD who do not respond to corticosteroids and/or other immunosuppressive therapies[12].

The safety of MSC therapy was confirmed in another phase 1 clinical trial conducted in patients with decompensated liver cirrhosis[13]. The objective was determined the safety of autologous BM-derived MSC transplantation. In this study, just like in the ones described above, MSCs were grown in culture medium with 10% of FBS for all the duration of expansion until the final harvest. Data suggest that there were no side effects in patients during follow-up[13].

FBS (final concentration of 10%) supplemented DMEM was also utilized in another study, which utilized antilogous BM-derived MSCs were utilized to treat 8 patients with old myocardial infarction. In

this case BM was obtained from the patients 2-3 months prior to intramyocardial injection, with BM MNCs isolated and expanded in DMEM with 10% FBS. The authors reported that the intramuscular or intracoronary injection of expanded MSCs was a safe and feasible procedure, and that there was a significant improvement in the cardiac function of MSC-treated patients during the 18 months follow-up[14]. No serious adverse events were reported by the authors in the in the peri-injection period.

In 2010 the researchers at the Copenhagen University Hospital launched MyStromalCell Trial, a prospective, randomized, double-blind, placebo-controlled, single center, Phase II study that investigated the effects of VEGF-A^{165} stimulated autologous Adipose Derived Stem Cells (ADSCs) in patients with chronic ischemic heart disease and refractory angina[15]. As previously reported, adipose tissue represents an abundant, accessible source of multipotent adipose-derived stromal cells with a great potential for *in vivo* differentiation into endothelial cells and cardiomyocytes. ADSCs were obtained from an autologous lipoaspirate and expanded in culture, in cell culture media supplemented with 10% FBS. The cells were passed once, until they reached 80-90% confluence, and stimulated to differentiate towards endothelial cells by culturing them for 7 days in VEGF-A^{165} stimulated medium containing 2% FBS. In preparation for infusion, cells were re-suspended in PBS supplemented with 0.1% Human Serum Albumin (HSA). The follow-up assessments were carried out after 4, 12 and 26 weeks, and at 1, 2 and 3 years, with no adverse effects reported. The data clearly demonstrated a reduction in the extent of infarct size in the left ventricle and left ventricle ejection fraction.

In 2012 Manoj et al conducted a systematic review and meta-analysis of clinical trials that utilized MSCs as therapy for a number of disease conditions that included ischemic stroke, Crohn's disease, cardiomyopathy, myocardial infarction, and graft-versus-host-disease (GVHD). The study was conducted to detect associations between MSC treatment and the development of acute infusional toxicity, organ system complications, infections, death or malignancy. 36 randomized controlled and uncontrolled clinical studies were included in the review[13]. Twenty-seven of the 36 studies cultured the MSCs in FBS, five in human serum (HS) and four did not report the source of serum uutilized. Based on the available data the authors concluded that the only toxicity reported was transient fever. No other evidence of increased susceptibility to infection following the administration of MSCs culture in media supplemented with FBS was reported. This suggested that clinical applications MSCs-based therapies, where cells were cultured and expanded in animal-sourced FBS MSC were safe[16].

FBS (final concentration of 10%) supplemented HEM's F12 medium was utilized to isolated and expand cardiac stem cells in another study that presented the evidence in support of FBS use in the manufacture of products for clinical applications. In the Scipio Trial - a phase 1, randomized, open label trial of autologous c-kit+ cardiac stem cells (CSCs) for the treatment of heart failure resulting from ischemic heart disease cardiac stem cells were reproducibly isolated from 1 g myocardial tissue that was harvested during cardiac surgery and expanded in culture in aHam's F-12 medium supplemented with 10% FBS. Following expansion c-kit+ CSCs were obtained by immunomagnetic sorting[17]. Data confirmed that infusion of 1 million autologous CSCs was not associated with any apparent adverse events at 1-year follow-up. Additionally, at 1 year follow-up intracoronary infusion of autologous CSCs was effective in improving left ventricle systolic function and reducing the infarct size[17].

In 2008 De Lima et al conducted a phase I/II trial to test the feasibility and safety of transplantation of CD133+ cord blood (CB) hematopoietic progenitors cultured in media containing stem cell factor, FLT-3 ligand, interleukin-6, thrombopoietin, tetraethylenepentamine (TEPA) and 10% FBS, in patients with advanced hematological malignancies. The CD133+ CB cells were expanded *ex-vivo* for 21 days, before infusion. The data demonstrated that the expanded cells were well tolerated with no infusion-related adverse events observed in any of the participating patients[18].

The data discussed above clearly indicates that MSCs have a number of potential applications, both in regenerative medicine and tissue engineering. Jung at al proposed that available MSC culture and expansion protocols that utilized FBS in a static adherent culture to achieve clinically relevant number of MSCs, were not highly reproducible, controllable or highly scalable. The authors came to the conclusion that these protocols did not meet the demands of regulatory agencies for high quality therapeutic cells required for expanded clinical applications. Hence, optimized manufacturing protocols should offer (1) optimized growth conditions, (2) monitor-

ing capacity of the primary and passaged cultures, (3) scalability. These processes should be able to produce large number of clinically acceptable cells in a rapid, safe, reproducible, and easily scalable manner[19-21]. In order to achieve this, the use of FBS in culture, expansion, and differentiation of a variety of stem cells needed to be addressed.

FBS SUBSTITUTES AND SERUM-FREE MEDIA

The supplementation of basal culture media with animal serum is essential for cell growth and stimulation of cell proliferation. FBS has been most frequently used to supply growth factors to culture medium because it is an extremely complex mixture of a large number of constituents, low and high molecular weight biomolecules with different, physiologically balanced growth-promoting and growth-inhibiting activities and is relatively readily available in what approximates a "clinical" grade. However, it should be noted that due to the fact that FBS remains poorly characterized, different batches of FBS show considerable qualitative and quantitative variation in their composition – growth factor and hormone activity, etc. – which seems to change from batch to batch. Hence, significant batch-to-batch variability does exist. Furthermore, FBS remains associated with safety issues that include bacterial and/or fungal contamination, transmission of prion or viral disease, anaphylactic reactions, and production of anti-FBS antibodies. In fact, regulatory authorities in a large number of countries, which include countries in the European Union, now prohibit the use of FBS for clinical applications[10]. At the same time, other countries such as Australia and US, i.e. Australian Therapeutic Goods Authority and FDA, allow the use of FBS for the production of clinical grade products, as long as the FBS is sourced from cattle in a country that is deemed free of bovine spongiform encephalitis[18]. These include, Central and South America, US, Australia and New Zealand.

To avoid the risk associated with the use of FBS and other animal-sourced supplements, the use of human-derived media supplements that include human allergenic or autologous serum, platelet lysate, human serum albumin, umbilical cord blood and placental serum (primitive growth factor rich tissues), etc. has been proposed[19-23]. The effect of human allogeneic (HS) and autologous serum (AS) from adult donors to enhance proliferation of MSCs with preservation of important cellular and functional properties remains controversial[20,21]. Results demonstrate that human MSCs proliferate much more rapidly in HS-supplemented culture media, compared to MSCs cultured with FBS, with chondrogenic or osteogenic differentiation capacity remaining the same between the two groups[20,21]. However, contradictory results using human serum have been reported. While the use of AS would minimize safety concerns widely associated with the use of FBS, some draw back exist. As large quantities of FBS are required to manufacture high quality, regulation-compliant MSC-based therapeutic products, it will be problematic to acquire large amounts of AS sufficient to generate clinically relevant numbers of MSCs. This is due to the fact that autologous serum from elderly patients may have deteriorated capacity to support cell growth, and the pool of age-appropriate donors is not sufficient to meet the current demand[10,20,21]. In addition, donor co-morbidities, such as diabetes, and injury-associated active inflammatory state could potentially influence the quality of the serum. Thus, it might be beneficial to utilize well-characterized allogeneic serum from a pooled, ABO-controlled, young, disease-free donor population[21].

Human platelet lysate (HPL) has recently become a more preferred human-derived media supplement product, and has been used in 10% of 47 clinical trials analyzed by Ikebe and Suzuki[7]. Platelet lysate can be easily obtained from apheresis products, as well as buffy coats of healthy volunteers and can be pooled from several healthy donors[10,24,25]. Immediately after collection, platelet products are frozen at –80°C and subsequently thawed so that growth factors included in platelets are released and platelet bodies are eliminated with subsequent centrifugation. As demonstrated previously, the properties of platelet lysate are based on the release of multiple growth factors that include platelet-derived growth factors (PDGFs), basic fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF), insulin growth factor-1 (IGF-1) and transforming growth factor (TGF-b), all of which have been demonstrated to improve proliferative capacity of MSCs[24,26]. While trying to investigate whether MSCs could be grown in HPL-supplemented media, Doucet at al demonstrated that compared to FBS-supplemented culture media, the latter was able to successfully promote MSC expansion, decrease the time required to achieve cell confluence, and increase clonogenic efficiency[26].

Bernardo et al confirmed these results by demonstrating that MSCs expanded in media supplemented with either 10% FBS or 5% HPL display

comparable cell purity, morphology, phenotype, and differentiation capacity. The expression of MSC-specific markers expressed on the cells cultured in HPL remained unchanged in all donors tested. Additionally, the authors demonstrated that clonogenic efficiency, proliferative capacity and immunoregulatory properties of MSCs was far more superior when cells were grown in a culture medium supplemented with 5% HPL compared to that with 10% FBS[24]. At the same time, the immunosuppressive capacity of the MSCs-HPL on alloantigen-induced lymphocyte proliferation was less evident compared to MSCs cultured in FBS[10,24]. Hofbauer et al[27] looked at the use of HPL as a potential replacement for FBS as a media supplement for culture of endothelial cells (ECs). The authors reported that expression of EC markers was similar for ECs grown in FBS and HPL. The fact that the use of HPL in culture media for the expansion of MSCs resulted in a decrease of MSC-associated *in vitro* immunosuppressive capacity was confirmed by Abdelrazik et al[28]. The authors also showed that while proliferation was greatly enhanced, MSCs cultured in HPL-supplemented media demonstrated an altered expression of MSC-specific surface markers[10,28].

Although considered relatively safe – compared to FBS – for human therapeutic applications, the use of human sourced supplements is still a matter of substantial debate. Product-specific concerns associated with the use of allogeneic human-derived media supplements. There is always a risk that any allogeneic human product may be contaminated with human pathogens that might not be detected by routine screening. Additionally, crude preparations of human blood derivatives are as poorly defined as FBS. Hence, there is an inherent variability associated with their use as media supplements for the manufacture of cellular therapeutics designated for clinical use, i.e. their ability to maintain consistent MSC growth and therapeutic potential. Additional data is needed before HPL is accepted as a standard replacement for FBS.

Concerns raised by the use of poorly defined FBS or human-sourced media supplements encourage the need for the development of serum-free and/or animal product-free cell culture media. At the present time, the importance of culturing mammalian cells in medium without animal-sourced serum supplements is well recognized. Consequently, the last two decades saw the rapid development of serum-free and/or chemically defined commercially available cell culture media. Chemically defined culture media may be of great benefit, as its composition can be defined to a great detail, reducing quantitative and qualitative variability and largely eliminating a potential source of microbial and infectious disease contamination. Additionally, the use of serum-free and/or chemically defined media can resolve the ethical dilemma associated with the world-wide use of the FBS, thereby reducing the number of calf fetuses required to meet the current demand for the FBS[29,30]. Once a particular "chemically defined" cell culture media is demonstrated to be efficacious across multiple cell types, it can be reproduced and its production scaled up with great precision. Of additional benefit is the fact that "chemically" defined media may help simplify the manufacturing process, and adept it to the cGMP environment required for the manufacture of clinically designated cellular therapeutics. With the identification, cloning, and recombinant production of essential growth factors and nutrients required by different cell types, a broad selection of chemically defined serum-free media for continuous cell lines as well as specific cell types in primary culture has been designed and is available[29].

At the same time, critical issues associated with the use of serum-free media do exist. The rate of cell growth in serum-free media is not always comparable to that of cells cultured with serum supplements, although Lindroos et al did report a higher proliferation rate of adipose derived stem cells (ADS) in serum-free media compared to ADS cultured in media containing serum[23,31]. One of the first groups who studied the influence of various growth factors and other supplements on MSCs cultured in serum-deprived conditions were Gronthos and Simmons[31]. They studied 25 different growth factors and found that the combination of IGF, PDGF BB and epidermal growth factors (EGF) together with dexamethasone and ascorbic acid led to superior MSC cell yields compared to other growth factor combinations or culture in serum-free media[31].

As *Ex-vivo* cultured, expanded, and differentiated cellular products have been demonstrated to be safe as a replacement therapy across several disease conditions, they've become more desirable. The transition from an expansion state to a differentiation phase under defined and controlled conditions could facilitate the production of such cellular therapeutics for clinical use and their commercialization. Translation of cellular therapies into the clinical setting requires compliance with cGMP quality and manufacturing standards. These products fall under the Health Service Act (HAS) 351

regulations established for extensively manipulated somatic and other cell products, as well as FDA regulations established for biologics and drugs.

Due to its rich content of growth factors, proteins, hormones, etc. and it's relatively low gammaglobulin content, FBS has become the most widely used supplement in cell culture media. There are clear advantages and disadvantages associated with the use of FBS. The advantages of using FBS in cell culture are as follows: (1) FBS represents a cocktail of most of the growth factors required for cell growth and proliferation; (2) FBS represents a universal cell growth supplement effective across most cell types; (3) the use of FBS-supplemented culture media eliminates the need for R&D necessary to formulate cell-specific growth media. The disadvantages of using FBS have been discussed elsewhere in this commentary; these lead to an increased risk associated with the administration of cellular therapeutics for clinical use that utilize FBS as part of their manufacturing process[29]. This is not ideal, especially in the view of the fact that cellular therapies for clinical use must be manufactured using established, reproducible and validated GMP manufacturing processes, and in compliance with the existing FDA, EU, World Health Organization (WHO), and other applicable regulations. Although the use of a FBS as a culture media supplement is still under discussion, at the present time it's widely utilized in research applications, as well as clinical scale production of a wide range cellular therapeutics[32]. This is due to the fact that effective alternatives with the same wide acceptability are still missing, and it is very often impossible to exclude the use of FBS without significant changes in cell culture efficiency.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

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