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Integrative review for replacement of fetal bovine serum by platelet-rich human plasma for culture and ex vivo expansion of human cells for advanced therapies

Revisão integrativa para substituição do soro fetal bovino por plasma humano rico em plaquetas para cultivo e expansão ex vivo de células humanas destinadas às terapias avançadas

Karla Menezes

Esther Rieko Takamori

Marcus Vinicius Telles Teixeira

Rosana Bizon Vieira Carias

Radovan Borojevic

Faculdade de Medicina de Petrópolis /FASE, Petrópolis, RJ, Brasil

* **E-mail:** karlamenezess@gmail.com

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ABSTRACT

Introduction: The advancement of clinical trials and cell therapies requires the replacement of fetal bovine serum by a product of human origin, able to sustain an expansion of human cells for research and cell therapy. **Objective:** This integrative review had as its main objective to evaluate different alternatives of cell culture supplementation free of animal products, called xeno-free cell cultures. **Method:** Fifty selected articles from PubMed published up to January 2018 in English or Portuguese were evaluated. **Results:** Platelet rich plasma (PRP) is considered to be a good alternative for supplementation of the cell culture media. PRP is obtained from blood and has a rich content released by activated platelets, capable of stimulating proliferation and differentiation of several cell types, both differentiated and the progenitor cells. The use of PRP in "xeno-free" cell culture systems has apparently no risk of genetic alterations of the cells, nor their contamination with pathogens. Advantages of its use include: 1) the possibility of using autologous serum; 2) reducing the risk of contamination; 3) easy preparation and 4) low cost of production. **Conclusions:** The use of discarded platelet concentrate in hemotherapy centers is a good alternative for the production of PRP, which will be used systematically in the culture of human cells. The challenge is to standardize this production process, in order to ensure the quality of the product to be used in advanced therapies.

KEYWORDS: Platelet-rich Plasma; Cell Culture; Cell Therapy; Clinical Trials

RESUMO

Introdução: O avanço dos ensaios clínicos e da terapia celular implicam na necessidade de substituição do soro fetal bovino por um produto de origem humana, capaz de sustentar a expansão de células humanas destinadas às pesquisas e terapias celulares. **Objetivo:** Esta revisão integrativa teve como objetivo principal avaliar diferentes alternativas de suplementação de cultura celulares livres de produtos animais, chamadas de culturas celulares *xeno-free*. **Método:** Foi realizada a análise de 50 artigos recuperados do PubMed publicados até janeiro de 2018 em língua inglesa ou em português. **Resultados:** O plasma rico em plaquetas (PRP) é considerado como uma boa alternativa para suplementação do meio de cultura celular. O PRP é obtido a partir do sangue, e possui um rico conteúdo liberado pelas plaquetas ativadas, capaz de estimular a proliferação e a diferenciação de diversos tipos de células, tanto diferenciadas quanto progenitoras. A utilização do PRP em sistemas de cultura celular *xeno-free* não oferece risco de alterações genéticas da população celular, tampouco sua contaminação com patógenos. As vantagens de sua utilização incluem: 1) a possibilidade de uso de soro autólogo; 2) redução de riscos de contaminação; 3) facilidade de preparo e 4) baixo custo de produção. **Conclusões:** O uso de concentrado de plaquetas descartados nos centros de hemoterapia é uma boa alternativa para a produção do PRP, que será utilizado sistematicamente na cultura de células humanas. O desafio é padronizar esse processo de produção, de forma a garantir a qualidade do produto destinado às terapias avançadas.

PALAVRAS-CHAVES: Plasma Rico em Plaquetas; Cultura Celular; Terapia Celular; Ensaios Clínicos

INTRODUCTION

Fetal bovine serum (FBS) is the most widely used supplement for the *ex vivo* expansion of human cells to be used in clinical trials and cellular therapies. Nevertheless, the guidelines of good manufacturing practices (GMP) of inputs for clinical use recommend replacing animal-derived products by chemically defined products or products derived from humans.

FBS is not a defined product. Because of its bovine origin, immune reactions against xenogeneic antigens in the receiving organism cannot be excluded. FBS proteins associate with the major histocompatibility complex (MHC) of class I in long-term cell cultures, leading to the proliferation of T cells in the cell culture, even in a setting using cultured autologous cells[1]. The glycoconjugates of bovine serum may be directly transferred from the medium to the cells and incorporated into the cell membranes of the cultured cells, remaining in its composition for up to 48 hours, which may cause an acute immune reaction. Pathogens and their derivatives, such as mycoplasmas, bacterial endotoxins, viruses and prions, are not necessarily eliminated from the culture medium containing bovine serum and they can be transferred to patients who will receive the cell transplant [2,3,4,5]. Furthermore, there are concerns about the imbalance between global demand and FBS supply from an ethical point of view and from the standpoint of animal well-being, since FBS is collected from bovine fetuses[6,7]. It is also important to consider the problem of FBS batch to batch variability, with qualitative and quantitative differences derived from geographical and seasonal influences. All these factors lead regulatory agencies to recommend the development of cell culture protocols free from animal products, called xeno-free cell cultures[6,7].

The challenge is to find a biological substitute of human origin that does not pose health risks and is able to support cell proliferation. The quantities of cells used in clinical trials or cell therapies are high and can reach or exceed 100 million cells per patient. This requires a high and long stimulation of cell proliferation *in vitro*[8]. Also, it is important to keep the cell profile throughout the *in vitro* expansion process, which can vary from a more immature stage up to a complete cellular differentiation, depending upon the quality of the culture medium and its supplements[8].

Different populations of human cells are evaluated in clinical trials. Adult cells, in advanced stages of cell differentiation, can be isolated from human tissues and applied in the treatment of specific diseases. They have a limited capacity of cell proliferation, and their clinical application is limited. Progenitor cells and/or adult stem cells, on the other hand, have a high potential of cell proliferation. They may originate in different cell lineages and are the subject of numerous studies. The most well evaluated type of stem cells in clinical trials in the world are the mesenchymal stem cells (MSC)[9]. There are currently 760 clinical trials evaluating the safety and efficacy of MSC treatment in different pathologies*. The main characteristics of the MSC include: adherence to plastic surfaces, fibroblastoid morphology, expression of surface markers (CD105, CD73 and CD90), ability to form colonies from only one cell (fibroblast colony-forming units, CFU-F) and to differentiate into cells of mesodermal and ectomesodermal origin[10,11]. MSC are easily expanded *in vitro* and are considered immunologically inert, which reduces the risk of cellular transplant rejection[12]. Most studies have used MSC isolated from human umbilical cord, peripheral blood, bone marrow or adipose tissue, but MSC may be isolated, *a priori*, from any human adult tissue[13].

This review study aimed to evaluate different xeno-free cell culture supplementation alternatives. In particular, we investigated the potential for utilization of human platelet rich plasma as a substitute for FBS in human cell cultures.

METHOD

This study was done as an integrative review. We evaluated 50 out of 75 scientific papers that addressed methods of xeno-free culture supplementation, comparing techniques and results. The research of scientific literature was done in documents of the Brazilian Ministry of Health and in the PubMed database for papers published in English or Portuguese until January 2018. Keywords: cell culture, xeno-free, platelet-rich plasma, plasma, platelet lysate, chemically-defined xeno-free medium, cell therapy, clinical trials, mesenchymal stem cells, progenitor cells, human cells, human serum.

RESULTS AND DISCUSSION

Alternatives for xeno-free culture systems

There are different supplements of xeno-free cell culture commercially available on the market. These manufactured products are standardized and do not interfere with the cellular profile[14]. However, some studies warn us of important biological changes in some human cells when cultured with commercial xeno-free culture media. MSC of human adipose tissue, cultured in xeno-free culture systems with manufactured products, showed a significant reduction in cell adhesion and loss of CD54 cell surface marker (ICAM-1). Variations in the expression of CD11a, CD14, CD10 and CD86 were also detected. These are related to the interaction of MSC with cells of the immune system, which may have affected their immunogenicity[15]. In addition, MSC are unable to form spheroids when cultured in three dimensions with some types of xeno-free culture media[16]. There is a change in the genetic profile of these human cells, with reduced production of anti-inflammatory cytokines and antitumor molecules[16]. It is believed that the therapeutic potential of MSC can be altered by the use of certain xeno-free commercial products[15,16]. There is a misconception that these manufactured products are chemically defined and totally devoid of animal products. In fact, they have growth factors in undefined amounts and can contain human or animal serum albumin[15].

Another option of xeno-free cell culture supplementation is the addition of growth factors to the culture medium. They may be synthetic or derived from animal or human tissue, used alone or combined as a cocktail[15]. The use of growth factors has been associated with increased cell proliferation[16,17] and may induce a specific cellular phenotype[16]. However, their applicability is limited because they are not able to sustain long-term cell expansion[15].

Human platelet derivatives applied in xeno-free cell culture systems

Products derived from human blood plasma are now considered as substitutes for FBS in xeno-free cell culture systems[18,19]. Pure blood plasma, collected from a donor in normal physiological conditions, usually contains small amounts of cell growth factors. However, the blood plasma has platelets that, despite having a relatively simple cyto-architecture, have an intricate and very well-organized content. This content is released only with the activation of platelets. This can occur under the effect of pro-inflammatory circulating factors, indicating the presence of an aggression or tissue irritation. Alternatively, an injury of the vascular walls can cause severe bleeding and needs to be resolved immediately. It exposes platelets to contact with the tissue extracellular matrix, mainly collagen and associated glycoconjugates, which immediately activate platelets, causing the release of their content. The first consequence is the onsite activation of the blood coagulation cascades, which interrupts the bleeding with the release of its granules and bioactive factors like serotonin, histamine, dopamine, calcium and adenosine.

Both inflammation and platelet activation by adhesion in injured tissues also signal the need to initiate tissue repair processes. This is the second function of activated platelets. They are the first blood components that arrive at the site of the lesion and release their granular content to promote tissue repair[20]. In this activity, platelets act as large reservoirs of enzymes, hormones, and growth factors. Several cell growth factors have been identified, among them: tumor growth factor (transforming growth factor -b-TGF-b), platelet-derived growth factor (PDGF), insulin-like growth factor I, II - IGF-I, IGF-II, fibroblast growth factor (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and endothelial cell growth factor. These growth factors and bioactive molecules produced by the platelets modulate and stimulate local cells to promote regeneration. This modulating ability is the main justification for using platelet content in a cell culture environment[21].

The platelet-derived products that are being evaluated in xeno-free cell culture systems are: 1) Pure Plasma (PP); 2) Platelet Rich Plasma (PRP); 3) Platelet Lysate (PL). PP and PRP are obtained through a peripheral blood centrifuging sequence. PP is obtained by only one centrifuging cycle, while PRP is obtained after two or more consecutive centrifuging cycles. PL, also known as Platelet Derived Hormones (PDH), is obtained by centrifuging followed by the addition of epinephrine agonist, and thereafter repeated cycles of cryofreezing and thawing and hypotonic shock to disrupt the platelet plasma membrane and release all its content[18,19].

In common, they all have the same biological components, that is, they have rich platelet content. However, there is great divergence in the nomenclature and methods of preparation of these biological products. There is no standard in the number of centrifuging cycles, ideal speed and temperature for isolating each of these products, nor is there consensus on a specific protocol for culturing each cell type[56]. The variations also include aspects like donor selection, collection process (apheresis or total blood donation), presence of additive solution, pathogen inactivation implementation, type of platelet activation, presence or absence of leukoreduction and consideration of ABO blood groups. All these factors may influence its applicability[19]. The use of PP, PRP or PL as human cell culture supplements has distinct effects on cell behavior and may influence migration, proliferation and differentiation of human cells differently (Table). The variability of the production process is a problem for the standardization of these biological products and should be a priority in the process of developing a xeno-free alternative of cell cultures[19].

Platelet Rich Plasma as a cell culture supplement

Among the different platelet products, PRP is considered the most promising supplement for culture of human cells intended for clinical use[5,19,21,27,32,40]. The PRP is obtained through a fresh blood centrifuging sequence aimed at increasing the platelet concentration in a small volume of the plasma itself [21]. The advantages of using PRP in xeno-free human cell cultures are: 1) the possibility of using autologous serum to prepare the cellular implant; 2) reduction of risks of cross-contamination and undesired immunological reactions of the patient; 3) presence of growth factors that stimulate cell proliferation and differentiation; 4) easy preparation; 5) low cost of production and 6) the possibility of allogeneic use for large scale cell culture.

The rich content released by the activated platelets present in PRP is capable of stimulating cell proliferation and the differentiation of various cell types, from adult cells to progenitor cells[22]. PRP was evaluated as a supplement of xeno-free culture in different types of adult human cells, such as: skin fibroblasts[41], gingival fibroblasts[38,39,40], periodontal ligament cells[39], osteoblasts[37], chondrocytes[27,31], myoblasts[28], tenocytes[26,29] and human meniscus fibrochondroids[57]. PRP gel has also been studied as a way of harboring cultured cells *in vitro*[41,42,43,44]. It is able to create a favorable environment for the formation of a three-dimensional (3D) cellular network[44] and provides suitable support for skin fibroblasts[41] and endothelial cells[42].

Overall, preclinical studies evaluated the ability of cell adhesion, migration, proliferation and differentiation upon the addition of PRP to the culture medium. There are no reports of tumor formation in any study[5,19,21,27,32,40]. The use of PRP favors cell adhesion on the culture substrate and stimulates migration through the reorganization of the cellular cytoskeleton[39]. PRP does not affect the potential for cell proliferation and, in some cases,

Table. Comparison between different products derived from human peripheral blood used as supplements for xeno-free cell culture.

Products	Acronyms	Method of production	Advantages	Disadvantages	Results on cell culture					Production of extracellular matrix proteins	Inflammation
					Cell proliferation	Cell phenotype	Cell differentiation	Cell migration	Senescence		
1) Pure Plasma	PP	Single blood centrifuging at low rotation and speed[9].	* The preparation of PP is easy, fast and cheaper than PRP and PL[22].	* Low concentration of platelets and growth factors in relation to PRP and PL[22].	* PP exhibits a similar effect to FBS in the proliferation of expanded human adipose tissue MSC *in vitro*[5,14].	* PP used as MSC culture supplement of human adipose tissue does not change its phenotype[5,14]	* PP does not alter its cell differentiation potential of human MSC[5,14], but osteogenic differentiation may be less favorable in PP supplementation than with FBS[23].	-	* Human MSCs expanded in allogeneic PP may enter into suffering and undergo apoptosis[24,25].	-	-
2.1) Platelet Rich Plasma	PRP	Two or more consecutive peripheral blood centrifuging cycles[9].	* PRP has a higher concentration of platelets and growth factors than PP[22]. * Supplementation with human PRP of human MSC culture does not cause the formation of tumors[26].	* A large volume of blood is required for the preparation of PRP[14]. * There is a biological variability between different samples of PRP[14].	* Cell proliferation of human MSC supplemented with PRP is 13.9 times greater than when they are cultured with FBS[27]. * PRP used as a xeno-free culture supplement increases proliferation of myoblast[28], tenocytes[29], chondrocytes[10,30,31], bone marrow MSC[10], human adipose tissue MSC[27,32,33].	* Supplementation of human MSC culture with PRP does not modify its phenotype, and does not cause chromosomal changes[27].	* PRP, used as a cell culture supplement, stimulates osteogenic differentiation of MSC[34,35,36], osteoblasts[37] and human dental pulp stem cells[38]. * PRP, used as a xeno-free culture supplement, does not modify the differentiation capacity of human MSC[5,19,21,27,31].	* PRP enhances the *in vitro* migration of chondrocytes[31], osteoblasts[31,37], human gingival fibroblasts[39,40] and human bone marrow MSC[10].	* PRP, used as a supplement for human MSCs, can slow the rate of cellular senescence[5,26,35].	* PRP, used as a cell culture supplement, stimulates the production of type II collagen and proteoglycans by subchondral MSC[10] and human tenocytes[29].	* PRP, used as a supplement for culture of human MSCs, preserves the immunoregulatory properties[5,21,19,26,27].
2.2) Platelet rich plasma in gel form	PRP gel	One or two consecutive blood centrifuging cycles and addition of calcium or thrombin for activation of the coagulation cascade[26,41].	* The PRP gel favors the formation of a three-dimensional (3D) cellular network[41,42] that mimics the *in vivo* environment and its cell to cell and cell to extracellular matrix signaling[43,44].	* The preparation of PRP gel is complex. It depends on the adequate concentration of platelets and requires a large volume of blood[9].	PRP gel stimulates the *in vitro* proliferation of human endothelial cells[42] and human fibroblasts[9].	* PRP gel promotes the formation of spheroids in MSC 3D cultures of human bone marrow[26], tenocytes[26], human fibroblasts[26,41].	* PRP hydrogel stimulates bone marrow MSC tenogenesis[26]. * PRP gel stimulates cell differentiation of human fibroblasts[41].	-	-	-	* The PRP hydrogel decreases the expression of inflammatory cytokines by MSC from bone marrow[26] and also from human skin fibroblasts[39].
3) Platelet Lysate	PL	Two consecutive blood centrifuging cycles, epinephrine addition, freezing, thawing and hypotonic shock[9].	PL has a higher platelet concentration than either PP or PRP[45,46,47,48].	* The PL preparation is complex, with great biological variability[9]. * The PL used in the culture of human MSC can induce ectopic bone formation[34].	* PL increases the proliferation of human MSC cultured in vitro[49,50,51,52,53,54].	* There are no chromosomal changes of the human MSC cultured in PL[49,50,51,52,54,55].	*PL, used as a supplement for culture of human MSC, retains its potential for cell differentiation [5,14,50,51,52,53,54], and can stimulate the osteogenic differentiation[34].	-	*PL, used as a supplement for culture of human MSC, decreases cell senescence[24,44,46].	-	* PL, used as a supplement for culture of human MSCs, preserves the immunoregulatory properties[53,54,56].

may increase the *in vitro* expansion capacity of these human cells[10,28,29,30,31]. The effect of PRP on cell differentiation varies according to the type of cell cultured. Myoblasts from human skeletal muscle do not differentiate in the presence of PRP in culture medium[28]. On the other hand, tenocyte progenitor cells are induced to differentiate into tenocytes and begin to produce collagen[29]. The same behavior is observed with chondroblast. PRP induces its differentiation in chondrocytes and promotes the formation of dense and compact cartilaginous tissue, rich in type II collagen and proteoglycans[10].

Another cell population that has been the target of many studies using PRP in xeno-free cell cultures are the MSC[14,27]. The risks of viral transmission from animal serum and the potential for immunological induction against bovine antigen in patients have been reported and raise concerns about the use of FBS for the preparation of MSC for patients undergoing clinical research[2,3,4]. Although serum-free culture medium is an optional condition for the preparation of MSC and a commercially available resource, the characteristics of the MSC appear to be variable according to the type of medium used[58,59].

Supplementation of the culture medium with human PRP has been recommended for the preparation of MSC for clinical protocols[14,19,60]. The reasons for this recommendation are: 1) the PRP added to the culture medium does not cause or stimulate the formation of tumors or genetic or phenotypical variations of the cultured MSC *in vitro*[27]; 2) PRP stimulates the proliferation of MSC without compromising their ability to differentiate[5,19,27,32] and their cellular immunophenotype[5,19,26,27]; 3) PRP can influence the differentiation of MSC to a specific lineage, in order to meet a clinical need [35,39]; 4) PRP can slow the rate of cellular senescence[5,26,35]; 5) PRP may influence the paracrine function of human MSC[61].

The use of autologous PRP is considered ideal for the preparation of human cells destined to clinical trials, since it does not offer risks of immune rejection for the patient who will receive the cellular transplant. Some studies have described that human cells can proliferate more quickly when supplemented with autologous plasma products than with allogeneic products[24]. There are some commercially available allogeneic products that can be used for large scale cell production. It is an economical option with limited variation in its composition, but it may pose alloimmunization risk[19]. The limitations that still exist on the application of human PRP in xeno-free cell cultures are the need for a large volume of blood for their preparation and the impact of their biological variability on cultured cells[22,33].

Blood banks are a strategic source of peripheral blood and its derivatives for the production of human PRP used for supplementation of xeno-free cell cultures. Also known as hemocenters, these blood banks are laboratories specialized in the storage and processing of peripheral blood that will be destined to clinical care. On average, 20 to 40% of the available blood is discarded for various reasons, mainly due to expiration of the shelf life. The index of losses of platelet concentrate in public blood bank reaches 33%. According to the general guidelines for blood banks, the platelet concentrate has a shelf life of five days. Because of this short shelf life, it is common to discard large amounts of this product after the recommended transfusion date[62,63,64,65,66]. In addition to avoiding waste, the use of blood and blood products in blood banks is a way to ensure that cell culture supplement products are of good quality, as they follow a processing pattern that minimizes microbial contamination and proliferation. They are also submitted to a detailed analysis in the collection act to verify the presence of possible infectious diseases, such as syphilis, HIV, hepatitis B and hepatitis C (recommendations described in Resolution of the Collegiate Board of the National Agency of Sanitary Surveillance RDC n. 24, of January 24, 2002).

Law n. 10.205, of March 21, 2001, and Ordinance n. 2.712, of November 12, 2013, specifically deal with blood, its components and derivatives, as well as the technical regulation of hemotherapy procedures, respectively[67,68]. They enable the use of this biological material, which was captured for therapeutic purposes and that had no programmed use, for the production of compounds and derivatives for other therapeutic purposes. The similar use could then be extended to the production of PRP for culturing of human cells and can stimulate the economic segment of biotechnology companies, encouraging investment in the production of research inputs. Blood components are considered as good quality raw materials. They have already been called "liquid gold"[62,66,69] due to the high profits of companies that invest in technological innovation.

CONCLUSIONS

This review study compared the results found in different xeno-free cell culture supplementation methods and found that human platelet rich plasma is the ideal candidate to replace FBS in cultured human cells for clinical research. There is no risk of genetic changes in the cell population[21,27,32] nor loss of their biological properties[5,19,27,32] and contamination with pathogens that could cause damage to the health of the patient who will receive the cellular transplant[27,32]. The challenges that exist for PRP to be effectively adopted in the preparation of human cells are: 1) standardizing the human PRP production process (number, speed, severity and centrifuging time), 2) determining the ideal concentration of human PRP to supplement the culture medium of different cell types; 3) establishing quality controls to detect possible exogenous contamination; 4) determining strategies to reduce biological variability; and 5) certifying qualified laboratories and researchers to offer these products to the domestic market. The development of clinical trials to evaluate the safety and efficacy of the transplant of human cells that have undergone platelet rich plasma supplementation during the *in vitro* cell culture and expansion process is necessary. These conditions should be studied as a priority of the scientific community and also evaluated by regulatory bodies that oversee clinical trials.

The platelet concentrate discarded at hemotherapy centers can be used for the production of human PRP. As long as it is frozen, this material discarded can be stored and processed under

suitable conditions. The purpose of its end use would be the cultivation of human cells under xeno-free conditions, for subsequent application in the culture of cells for advanced therapies. The competent authorities shall establish the handling parameters and the quality controls that are necessary to release the subsequent use of this product in advanced therapy procedures.

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Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.