

WOUND HEALING/EXPERIMENTAL

Fetal Bovine Serum Substitute: Implication for Their Use in Translating Adipose-Derived Stem Cells from Bench to Bedside (Review)

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Abstract: Recently, cell based therapy become a popular topic in research fields. This therapy offers great potential to treat many diseases. Adipose-derived stem cell (ASCs) is known to have the special characteristic due to its abundant availability and easily harvesting. Then it rapidly advanced into clinical trials for treatment of a broad range of medical conditions. Before translating ASCs from bench to bedside, this process is facing other problems through fetal bovine serum (FBS) elimination. Animal-derived serum is not a suitable option for ASCs expansion regarding the possibility of viral or bacterial infection and immune reaction. By replacing FBS with other non-animal serum which gives the same effect as FBS, may significantly enhances the safety and quality of ASCs. Pooled human AB serum (AB-HS), human serum (HS), pooled human plasma lysate (PL), umbilical cord serum (UCS)/placental serum (PS), thrombin-activated platelet rich plasma (tPRP) or serum free (SF)/xeno-free (XF) have been considered as FBS alternatives and some already applied in clinical trials with human cells. This article focuses on a prospective alternatives serum through the elimination of FBS.

Keywords: *Adipose-derived stem cells, cell based therapy, fetal bovine serum*

Abstrak: Akhir-akhir ini, terapi berbasis sel menjadi suatu topik yang populer di kalangan peneliti. Terapi ini menawarkan suatu potensi untuk mengobati berbagai penyakit. Sel punca derivat lemak diketahui memiliki keistimewaan karena jumlahnya yang tersedia banyak dan kemudahan untuk mengambilnya. Kemudian secara cepat hal ini berkembang ke dalam studi klinis sebagai pengobatan terhadap berbagai kondisi medis. Sebelum mentranslasi sel punca derivat lemak ini dari meja laboratorium ke pasien, proses ini menghadapi masalah lain yakni bagaimana menghilangkan serum fetus sapi. Serum yang merupakan derivat hewan merupakan pilihan yang buruk untuk ekspansi sel punca derivat lemak karena risiko terinfeksi virus, bakteri ataupun terjadinya reaksi imun. Dengan mengganti serum fetus sapi dengan serum nonhewani lainnya yang memberikan efek sama maka diharapkan dapat meningkatkan keamanan dan kualitas dari sel punca derivat lemak. Serum antibodi manusia, serum manusia, lisat plasma manusia, serum tali pusat/plasenta, plasma kaya platelet yang teraktivasi thrombin dan serum bebas/xeno bebas telah dipertimbangkan sebagai alternatif serum fetus sapi. Beberapa telah diaplikasikan pada studi klinis yang menggunakan sel manusia. Artikel ini berfokus pada prospek serum alternatif tanpa menggunakan serum fetus sapi.

Kata Kunci : *Adipose-derived stem cells, cell based therapy, fetal bovine serum*

Recently, regenerative medicine becomes a popular topic in many research fields. Many diseases currently have new hope to be cured. Naturally, human body provides its own regenerative system through the stem cells. This understanding leads many researchers to focus in stem cells and their probability properties as regenerative medicinal product. Cell based therapy is the administration of viable somatic cells¹ to help body to repair or regenerate tissue defect that associated with age, life style, environmental exposure²,

congenital or after trauma. Human cell-based therapy products should fulfill these following characteristics³: 1) *they contain viable human cells (maybe self-renewing stem cells, progenitor cells or terminally differentiated cells) of allogeneic or autologous origin undergoing a manufacturing process;* 2) *the cells may be combined with non-cellular components;* 3) *the cells may be genetically modified.*

Fat tissue is abundant, easily harvested⁴ and a source of adipose-derived stem cells (ASCs) both uncultured and cultured⁵. Therefore, ASCs are expected as

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promising candidate for cell-based therapy. But therapeutic dose of stem cells require a large number of cells. Thus, ASCs need to be expand before the clinical application. Standard cells expansion using fetal bovine serum (FBS) and other animal origin culture reagents is not an option in human clinical cell therapy due to several safety issues, such as immune reactions and infection⁶. Therefore, a safe and rapid expansion protocol should be established employing FBS substitute serum for cell based therapy.

Mesenchymal Stem Cells (MSCs): Phenotype and Differentiation Potential

MSCs were first isolated as fibroblast colony-forming units (CFU-Fs) or bone marrow stromal cells by Friedenstein and colleagues in 1968⁷. MSCs consist of number of stem cells with self-renewal ability and multi-lineage differentiation capacity into adipocytes, osteocytes, chondrocytes, hepatocytes, neurons, muscle cells and epithelial cells.

In undifferentiated state, MSCs show spindle shaped and fibroblast-like appearance⁸. Minimal criteria to define MSCs by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy are⁹: 1) *plastic adherence ability*; 2) *lack of hematopoietic markers, such as CD 45, CD 34, CD 14, CD 11b, CD 79 α , CD 19, or HLA-DR*; 3) *tripotential mesodermal differentiation potency into osteoblast, chondrocytes, and adipocytes*; and 4) *immunomodulatory capability*.

Adipose Tissue as Source of ASCs

Adipose tissue has been considered as a metabolic and sex steroid reservoir¹⁰. Basically, normal human adipocytes are typically 70 to 90 μ m in diameter and increase in size and number in obese people. Adipocyte structure is highly vascularized tissue¹¹ and surrounded by heterogeneous cell population which latter is termed stromal vascular fraction cells (SVF). SVF contain ASCs^{7,12}.

Clinical studies examining subcutaneous adipose tissue from 12 to 50 donors, have found

that ASCs capacity of proliferation and differentiation were decreased as age increased¹¹. ASCs isolated from women at different reproductive stages (pregnancy, premenopause and menopause) showed their consistency in cell differentiation capacity. Furthermore, the proliferation rate of ASCs after estrogen treatment remained unchanged¹⁰.

SVF versus ASCs

ASCs can be isolated from liposuction or through resection of adipose tissue. As heterogeneous population¹³, SVF or passage zero (P0) ASCs expressed hematopoietic markers [the common leukocyte antigen (CD45), the monocyte/macrophage markers CD11a, CD4, MHC class II DR histocompatibility antigen, and the costimulatory molecule CD86]. The presence of these potentially immunogenic cells could induce an allogeneic-rejection response and may be a source of pro-inflammatory cytokines¹⁴. These populations were disappeared by passage 1 to 4. ASCs expressed the mesenchymal stem cell markers (CD10, CD13, CD29, CD44, CD54, CD73, CD90, CD105, CD106, CD117 and STRO1) and negative expressed for hematopoietic markers (CD14, CD16, CD34 CD45, CD56, CD61, CD62E, CD104) or endothelial markers (CD31, CD144 and von Willebrand factor) and their levels did not change with passage^{15,16}.

ASCs in P1-P4 cells were significantly suppressed T cell proliferation in the mixed lymphocyte reaction (MLR), but not in the SVF population^{14,17}. Then generally, P1-P4 ASCs cells are not immunogenic. These changes in immunophenotype correlated with the level of immunogenicity displays by human ASCs in MLR. Additionally, ASCs suppressed the proliferative response of T cells to allogeneic stimulator cells, which infers that it will be possible to transplant ASCs across traditional histocompatibility barriers¹⁴. The other interesting characteristic of ASCs is their ability to migrate to areas of tissue injury; as a consequence, these cells can be used locally or systemically in cell based therapy. In ASCs migration capacity study showed that platelet

derived growth factor-AB (PDGF-AB), transforming growth factor- β 1 (TGF- β 1), and tumor necrosis factor- α (TNF- α) have the more effective chemoattractant activity. Pre-stimulation with TNF- α increased the migration activity of ASCs compared to unstimulated ASCs. ASCs were found to express CC chemokine (CCR1, CCR7), and C-X-C chemokine (CXCR4, CXCR5, CXCR6), and growth factor (epidermal growth factor-R, fibroblast growth factor receptor1, TGF- β receptor2, TNF receptor superfamily member 1A, PDGF receptor A and PDGF receptor B) at protein and mRNA levels. These results show that the migration capacity for ASCs is controlled by various growth factors and chemokines. Further, modulation of the homing capacity for ASCs could stimulate their movement into injured sites in vivo when administered intravenously¹⁸. These results proved that hASCs show a potential in cell based therapy.

Scientific considerations in replacing Fetal Bovine Serum

FBS is still the most widely supplemented serum used for cell culture even though it is not a suitable option due to patient safety^{19,20}. FBS contains xenogeneic proteins and associated with the possible contamination of viruses, prions, bacteria, nanobacteria, mycoplasma, yeast, fungi, and endotoxin. Some of them are impossible to remove from the serum. For a single injection of 100 million hMSC grown in FBS would carry about 7-30 mg internalized xenoprotein (N-glycolyneuraminic acid). Repeated administration of hMSCs contaminated by xenoproteins may cause immunologic rejection of the injected cells and may also lead to more serious complications such as autoimmune reactions against one's stem cells^{19,20}. Spees et al, were successfully removed xenogeneic proteins from bone marrow stromal cells (BMSC) expanded in 20% fetal calf serum (FCS) by incubating the cell samples in autologous human serum supplemented with 10 ng ml⁻¹ epidermal growth factor (EGF) and 10 ng ml⁻¹ basic fibroblast growth factor (bFGF) for 6 days²¹.

Although these two studies showed success results in removing xenogeneic antigen but further problems such as immunogenicity becomes usage consideration of FBS since a trial reported anaphylactic or arthus-like immune reactions in patients following the infusion of lymphocyte grew in FBS-media²². Another clinical studies using FBS-cultured hMSCs performed on 2000 patient have no reports on immediate adverse affect or infusion toxicity but late complications and rare unfavorable results occurred after long time follow up^{6,23}.

Alternatives Serum to FBS

Serum is a buffer and protection agent against "cytotoxic" agents by unknown mechanisms²⁴. The choice of serum gives affect on the health, quality of the cells growth⁶ and quantity of cells number. Pooled human AB serum (AB-HS), human serum (HS), pooled human plasma lysate (PL), umbilical cord serum (UCS)/placental serum (PS), thrombin-activated platelet rich plasma (tPRP), and serum free (SF)/xeno-free (XF) have been considered as alternatives for animal serum and some already applied in clinical trials with human cells^{6,24-26}. All these serum alternatives FBS have their drawbacks and advantages (Table 1). Bieback et al demonstrated that ASCs and BMSC differed in their demand for human blood-derived supplements. Pooled human PL was found to be the most suitable FBS substitute in BMSC expansion. On the contrary, both HS and tPRP, but not pooled human PL, were efficient in expanding ASCs²⁵.

These followings are the important criteria as an efficient medium alternatives¹⁹ : 1) *consistent in the concentration of the constituents*, 2) *absence of contaminants*, 3) *low cost*, 4) *longer shelf-life*, 5) *ready/easy availability*.

Human Serum (HS)

Blood serum was derived from plasma with removal of clotting factors. It contains 60-80 mg protein/ml besides various small molecules including salts, lipids, amino acids and sugars. The major protein constituents of serum include albumin, immunoglobulins,



transferrin, haptoglobin, and lipoproteins. Human serum also contains many other proteins that give effects in cells culture. It is estimated that up to 10,000 proteins may be commonly present in serum, most of which would be present at very low relative abundances²⁷.

Autologous human serum (autoHS) is a primary option for ASCs clinical application, which can be donated by the subject at the time of tissue collection. But sometimes autoHS may not be available in large quantities for cell expansions²⁴. AutoHS induced BMSC showed faster proliferation but less in differentiation capacity. This also proven on ASCs expansion^{6,26}. BMSCs supplemented 10% alloHS were dramatically different from autoHS. Fewer cells attached and formed colonies, and those cells never reached 60% subconfluence²⁸. So far no allogeneic antibodies against HS are found in patients that have received cell transplantation with BMSCs expanded in alloHS⁶. Different results in these studies might be caused by endogenous factors in HS. High variability in platelet derived soluble factors contained-HS may play a role on ASCs proliferation and differentiation. Our previous study was shown that PDGF AB, PDGF BB and TGF B1 concentration give significant effect in both ASCs proliferation and differentiation (not yet published). High concentration of PDGF AB (>15ng/ml), PDGF BB (>2,5ng/ml) and TGF B1 (>25ng/ml) contained HS showed superiority on ASCs proliferation and differentiation capacity than FBS. We proved that alloHS could be used as FBS substitute (Picture 1).

There is no difference in surface marker expression among ASCs cultured in FBS versus alloHS. Both of them showed strong expression in CD10, CD13, CD29, CD44, CD90 and CD 105, moderate expression for CD9, CD34, CD49d, CD166, HLA-ABC, hFSP and STRO-1, and lacked expression of hematopoietic and angiogenic cell lineages (CD31, CD45, and CD106) , HLA-DR^{6,25}. In multilineage differentiation capacity ASCs cultured in FBS exhibited a greater capacity for early osteogenic differentiation than ASCs cultured in alloHS. In the contrary, the presence of alloHS showed significant up regulation of adipogenic markers

α P2 and peroxisome proliferators-activated receptor γ (PPAR γ) compared to FBS. Spontaneous adipogenic differentiation was seen in the osteogenic induction cultures in alloHS. Chondrogenic differentiation capacity was equal between FBS and alloHS^{6,26}.

Human AB Serum (AB-HS)

Pooled human AB serum has been identified as an alternative medium to FBS. ASCs grown in AB-HS displayed a fibroblast-like morphology in smaller size and more spindle shaped^{25,29}. Interestingly, ASCs culture in FBS lost their spindle-like shape starting at passage 4 whereas AB-HS cells retained their spindle-shaped morphology throughout long-term culture, assuming spherical morphology only at the end of their proliferative phase after 40-50 population doublings²⁹. Furthermore, ASCs culture with AB-HS had decreased adhesion potential compared with FBS. ASCs culture in ABHS demonstrated a significantly higher fold expansion rate compared to FBS between passage 2 and 6. On the contrary, ASCs culture in FBS exhibited a slow but continuous proliferation²⁹. The differentiation capacities for FBS and AB-HS were comparable and maintained throughout long-term culture^{25,29}. There was no significant difference in surface markers expression for both FBS and AB-HS except for CD45/CD14. Positive side population of CD45/CD14 for AB-HS compared to FBS could be due to cytokine such as hemofiltrate CC chemokine (HCC-4) and Flt-3-ligand, which are present in serum and chemoattractants for monocytes²⁹. This pooled AB serum as the best choice because of the ease of preparation and substance such as heparin and thrombin are not included.

Human Platelet Lysate (HPL)

Human platelets have granules containing cytokines and growth factors³⁰. The properties of PL are based on the release of multiple growth factors on platelet activation³¹. PL is enriched in PDGF-AB, TGF- β 1, TGF- β 2, bFGF, PDGF α , PDGF β ³². Doucet et al, introduced a platelet-lysate protocol by using

only samples containing about 1×10^9 platelets/ml and frozen the samples at -80°C to obtain a PL containing platelet-released growth factors. PL products were the thawed and harvested to be used in culture as FCS substitutes³³. The levels of PDGF in PL might be expected to depend on number of platelets involved but Poloni et al, found there was no significant correlation between platelet count and growth factor levels³⁴. PL was proven to be the best alternatives serum in BMSC culture but not in expanding ASCs²⁵ due to difficult handling and preparation²⁹. In contrary, another study found that ASCs grown in PL exhibited higher cell population doubling time than ASCs culture in FBS. Regarding the high cells proliferation rate, the adipogenic induction was performed in culture supplemented with only 2.5% PL²⁴. The different results presented in PL studies could be explained by PL preparation. Theoretically, the efficacy of PL in promoting in vitro MSC expansion is attested by its growth factor content and the expression of their cognate receptors at the MSC membrane surface. Differences in the concentration of growth factors in PL are noted in particular for PDGF-AB and TGF- β 1 cause by using different PL production processes (apheresis vs. multi-step centrifugation separation of whole blood). Furthermore, the presence of growth factors in the PL supernatant is related to the efficiency of their release from platelet granulations. Degranulation is a dynamic process that could be influenced by some factors such as the storage conditions, number of PL freeze and thaw cycles before their use³¹.

Thrombin-activated platelet rich plasma (tPRP)

Platelet rich plasma (PRP) is blood plasma that has been enriched with platelets. PRP contains several growth factors and cytokines that released through degranulation of alpha granules by adding thrombin and calcium chloride. Platelet activation with thrombin closely imitates the physiologic activation of platelets, ensuring the bioactivity of secreted growth factors³⁶. The growth factors and cytokines present in PRP include PDGF,

TGF β , fibroblast growth factor (FGF), insulin like growth factor-1 (ILGF-1), insulin like growth factor-2 (ILGF-2), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), interleukin 8 (IL8), keratinocyte growth factor (KGF) and connective tissue growth factor (CTGF)³². To date, only few study using tPRP as ASCs growth medium have been published. In serial studies, Kacaomer et al found that thrombin was the optimal agent for releasing platelet factors into plasma²⁹. ASCs in tPRP culture conditions demonstrated a significantly higher fold expansion rate compared to FBS but no difference in characterization of surface marker expression. Cytokine profile comparison between AB-HS and tPRP showed, only four chemokines such as angiogenin, PAPC (pulmonary and activation-regulated chemokine), HCC-4 (hemofiltrate CC chemokine4) and IL-13 receptor α 2 had significant higher signal in AB-HS than in tPRP. Conversely, another five factors had a higher signaling intensity in tPRP compared with AB-HS; MCP-2 (monocyte chemoattractant protein 2), GRO (growth related oncogene), uPAR (urokinase plasminogen activator receptor), ICAM-2 (intracellular adhesion molecule 2) and LAP (liver activator protein). So far, ASCs grown in tPRP decreased in adhesion potential²⁹.

Human umbilical cord serum (UCS) / human placenta serum (HPS)

Umbilical cord serum derived cord blood contains higher growth factors than adult blood serum, such as erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF) and colony stimulating factor 1 (CSF-1), stem cell factor (SCF), IL-3, and IL-6. All these factors have been known can promote stem cells proliferation. Cord blood serum is safe serum that takes no risk of xenogeneic antigen, lower immunogenicity and rich content of the growth factors³⁷. HPS contains placental growth factors that have been known to have a most powerful affect on MSCs proliferation³⁸. UCS and HPS greatly increased ASC proliferative activity 2-3 folds. Moreover, ASCs grown in HPS reached

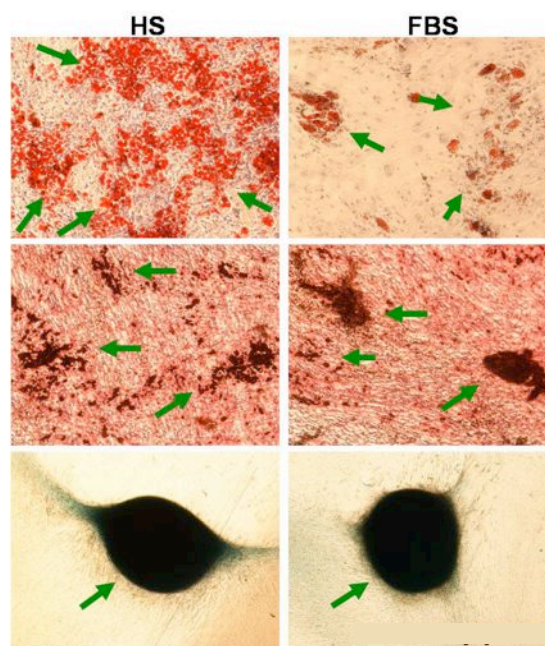


Figure 1: ASCs culture in induction medium supplemented human serum showed comparable result to FBS in differentiation capacity. In adipogenesis, ASCs culture in induced medium supplemented HS (left upper stand) showed higher density of positive Oil red O staining cells (as pointed by green arrows) than FBS (right upper stand). Osteogenesis results were shown the similar effect between HS (left middle stand) and FBS (right middle stand). Mineral deposition was detected by von kossa staining as black colour (pointed by green arrows). HS demonstrated superiority than FBS in chondrogenesis. ASCs cultured in chondrogenic induced media supplemented HS (left lower stand) perform larger cartilage nodule than FBS (right lower stand). The presence of sulfate glycosaminoglycans was detected by Alcian blue staining. Positive Alcian blue staining nodules

100% confluency after three days and continued its proliferation without contact inhibition. There was no difference in differentiation capacity between ASCs culture in FBS and HPS or UCS^{39,40}.

Xeno free medium (XF)

Xeno free medium perhaps is a safer and less variable option in supports ASCs proliferation and differentiation. Lindroos et al introduced StemPro XF (SP XF) expansion protocol for ASCs by supplementing the SP XF with LipoMAXTM. The SP XF medium formulation displayed a higher proliferation rate compared with FBS and HS while still maintaining surface marker expression profile and multipotentiality compared with FBS and HS²⁶. Hence, a defined serum free media formulation is one step closer to develop a safer clinical cell therapy but the presence of growth factors in medium is essential for cell

proliferation. Therefore, further investigation in preclinical safety and efficacy are necessary.

SUMMARY

ASCs based therapy almost becomes the new treatment for many diseases. However, translating ASCs from bench to bedside is facing many problems that need to be solved one by one Replacing FBS is the first priority for future cell based therapy application. Standard protocols and quality control contributed to the effectiveness of those serum alternatives are required. Finally, continuous research would definitely bring us in ongoing search for the best ASCs culture condition to get the cell therapeutic dose for clinical application of ASCs based therapy.

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