1. Introduction

"Research is to see what everybody else has seen, and to think what nobody else has thought"

- Albert Szent-Gyorgyi

(Hungarian Biochemist, 1937 Nobel Prize for Medicine, 1893-1986)

The cell is the small, basic, structural and functional unit of life. The cells are of various types. Cells taken from animal or animal tissue will continue to grow if they are provided with nutrients and growth factors needed for their survival. This process is called Cell Culture. In this process every cell behave as an independent unit. The cell divides by mitosis and its proliferation continues unless limited by some parameters like nutrient depletion, contact inhibition etc. This culture process occurs *in-vitro* (in glass) as opposed to *in-vivo* (in life) (Alberts et al., 2002).

Tissue culture was first initiated in 1900s as a method to study the behaviour of animal cell (Harrison, 1910). Initially the cells were cultured from un-disaggregated tissue fragments in which the cell growth was restricted to the outgrowths from the primary tissue (Carrel, 1912). The primary explant culture dominated the first half of 20th century. The second half of the 20th century has given facts & expansion in this area and was made available with the culturing of dispersed cells from tissue (Freshney, 1994).

Animal cell culture is an essential laboratory technique for the study of biochemical & physiological processes. It finds its applications

i) to investigate the normal physiology or biochemistry of cells. That is, metabolic pathways of a cell can be investigated by applying radio labelling of cells followed by analysis.

ii) to test the effects of compounds on specific cell types. Such compounds may be metabolites, hormones or growth factors. Similarly, potentially toxic or mutagenic compounds may be evaluated in cell culture.
An attempt to use Soybean protein as an alternate for Fetal Bovine Serum in animal cell culture

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iii) to produce artificial tissue by combining specific cell types in sequence. This has considerable potential for production of artificial skin for use in treatment of burns.

iv) to synthesize valuable biological products from large-scale cell cultures. The term biological encompass a broad range of products from living things or its basic unit cell that include specific proteins or viruses that require animal cells for propagation. The number of such commercially valuable biologicals has increased rapidly over the last decade and has led to the present widespread interest in animal cell technology. Proteins that are present in minute quantities *in-vivo* can be synthesized at a large scale by growing genetically engineered cells *in-vitro* (Butler, 2005).

Cell culture technique is preferred because; conditions can be controlled to allow some degree of consistency & reproducibility in cell growth. This technique also helps in characterization of cells across several generations and to maintain homogeneity. Cells are exposed directly to lower and defined concentrations of reagent thereby less reagent is needed *in-vitro*, providing economic feasibility. This allows the delivery of specific experimental compound of a particular concentration and duration to the actual site of action. This develops the histotypic and organotypic models which in turn increases the accuracy of *in-vivo* modelling (Freshney, 2010).

Successful growth and maintenance of animal cells *in-vitro* requires a culture condition that mimics *in-vivo* condition. The influence of the environment on the culture is expressed via

i) the nature of the substrate on or in which the cells grow *i.e.* solid or plastic or other rigid matrix; semisolid, as in a gel such as collagen or agar; or liquid, as in a suspension culture

ii) the degree of contact with other cells

iii) the physicochemical and physiological conditions of the medium

iv) the constitution of the gas phase

v) the incubation temperature. The provision of the appropriate environment, including substrate adhesion, nutrient & hormone or growth factor concentration, and cell interaction, is fundamental to the expression of specialized functions (Alberts *et al.*, 2002).
The cell culture media is the most required single factor for cultivation of animal cells. Earlier cells were cultured in natural media based on biological fluids like plasma, lymph clot, chick embryo extract and serum. Sometimes tissue extracts were also used. The propagation of cell culture and demand for larger amount of media has led to the use of chemically complex artificial liquid media. This media is suitable for the growth of cells in-vitro for several generations. This cell culture provides a microenvironment for cell metabolism, nutrients, cell growth & proliferation and all the complex chemical substances required by the cell that cannot be synthesised by it. This is called as the basal medium. The basal medium contains all the basic low molecular weight components such as basal salt, energy sources, amino acids, vitamins, pH indicators, buffering system, trace elements etc.

The chemical medium started with Ringer’s solution to MEM and its various modified forms. Each one has its own chemical composition and the chemical nature of it varies with the type of cells and purpose. Few of them are Eagle’s Basal Medium (Eagle, 1955) and subsequently, Eagle’s Minimal Essential Medium (MEM) (Eagle, 1959), DMEM (Dulbecco and Freeman, 1959), or increasingly, RPMI 1640 (Moore et al., 1967). A popular compromise for many laboratories is a mixture of complex medium, such as Ham’s F12, with higher amino acid and vitamin concentrations.

However these biosynthetic precursors for cell anabolism, catabolic substrate for energy metabolism and inorganic ions alone cannot support the cell growth by themselves. Hence there is a necessity to supplement the media with serum & growth factors. Therefore a complete medium contains two distinct part i) a basal medium which satisfies cellular requirements for growth containing chemical substances that enters a cell and is used as either a structural component or as a substrate for biosynthesis or energy metabolism or in a catalytic role and ii) a set of supplements that satisfies other cellular requirements & permits cell proliferation in the basal medium. The supplementation could be undefined additives like serum and other biological fluids.

Serum is a blood component devoid of RBC, WBC and clotting factor. Serum includes all proteins except those used in blood clotting (coagulation) and all the electrolytes, antibodies, antigens, hormones, and any exogenous substances like microbes and drugs (Wang, 2002). It is collected as supernatant of clotted blood. It is obtained commonly from any species of animal; bovine (fetal or adult), equine (horse), calf (fetal or adult) etc. Human serum is sometimes used in conjunction with some human cell lines, but it needs to be
screened for viruses, such as HIV and hepatitis B. Horse serum is preferred to calf serum by some workers, as it can be obtained from a closed donor herd and is often more consistent from batch to batch. Horse serum may also be less likely to metabolize polyamines, due to lower levels of polyamine oxidase. Polyamines are mitogenic for some cells (Kaminska et al., 1990). Serum is an effective growth promoting supplement for most cell types. This is because of its complexity, multiplicity of growth promoting, cell proliferating, nutritional factors and high content of embryonic growth factors. Serum is also a source of minerals, lipids, and hormones, many of which may be bound to protein.

In spite of all the above merits serum has demerits also. It is chemically undefined or ill-defined compound and variations between batches can result in inconsistent promotion of cell growth. It is expensive and accounts 50% of cost of the formulation. So use of serum is an important consideration in large scale culture. The total proteins in serum also interfere during separation and purification of desired protein product of interest, for example specific immunoglobulins. It may have endotoxins and other adverse factors. The use of animal derived serum / protein in mammalian cell culture may cause an infective risk by infectious prions, viruses, mycoplasma, bacteria and fungi. Moral concerns also exist regarding the application of FBS in cell culture. Recently care is also taken about the welfare of donor fetus during harvesting of fetal blood. FBS is commonly harvested by means of a cardiac puncture without anaesthesia (Brunner et al., 2010). Fetuses are exposed to pain & discomfort and therefore current procedure of fetal blood harvest is considered to be inhumane.

In order to address these facts studies to develop a biopharmaceutical product on the substitution or removal of animal derived component from the cell culture media is under process. One approach followed to reduce the serum requirement is by supplementing culture media with processed serum products. Controlled process serum replacement (CPSR) are prepared by process that yield defined products with much higher batch to batch consistency than serum. CPSR products are derived from bovine plasma and have lower protein and endotoxin levels than serum. Serum may be fortified with mitogen, growth factor, hormones, proteins & protein stabilizers and trace elements. Such fortified serum can often be used at a much lower concentration than natural serum.

From the breaking dawn of 21st century, serum and feeder cells have been replaced with various biological components. These components include Epithelial Growth Factor (EGF), Fibroblast Growth Factor (FGF) (Levenstein et al., 2006), noggin (Xu et al., 2005),
activin (Xiao et al., 2006), insulin, transferrin, hydrocortisone, Bovine Pituitary Extract (BPE), etc. (Martone et al., 2005). As these growth factors may increase the cost of the cell culture media, proteins and protein hydrolysate from animal products (peptone) and microbes (yeast extract) have been used as supplement in basal medium (Castle and Robertson, 1999). These supplements are reported to act as medium additives or replacement for FBS and other bovine extracts.

Similarly, proteins and protein hydrolysates from plant products have been used in cultures of hybridomas (Franèk et al., 2000), keratinocytes (Lee et al., 2008), CHO cells (Chun et al., 2007), primary rat hepatocytes (Hamel et al., 2006), BHK21 cells (Heidemann et al., 2000) and insect cells (Kwon et al., 2005). Such plant derived serum alternatives are called as vegetal serum. This is derived from chick pea (Girón-Calle et al., 2008), aloe (Tudose et al., 2009), wheat and rice gluten (Franèk et al., 2000) etc.

This led to the finding of many commercially available serum supplements like knockout serum replacement (KSR) (Kubikova et al., 2009), Serum replacement (SIGMA®) (Groh et al., 1996) and VegetaCell (Invitrogen™) (Kunova et al., 2010).

In addition to culturing of cells in-vitro, stem cells were also cultured in an artificial environment i.e) in-vitro stem cell culture using plant derived culture medium. Stem cells are terminally undifferentiated and are therefore able to produce cells of other types. The classical definition of a stem cell is that it possesses three properties.

- **Self-renewal** - the ability to go through numerous cycles of cell division while maintaining the undifferentiated state.
- **Potency** - the capacity to differentiate into specialized cell types. In the strictest sense, stem cells are to be either totipotent or pluripotent i.e to be able to give rise to any matured cell type. Multipotent or unipotent progenitor cells are sometimes referred to as stem cells.
- **Homing** - whenever the stem cells are given in-vivo they have a tendency to get along with that tissue or organ accordingly.

Here, potency specifies the potential of the stem cells to differentiate into different cell types.

Until recently, scientists primarily worked with two kinds of stem cells from animals and humans: embryonic stem cells and non-embryonic "somatic" or "adult" stem cells. The Bone Marrow (BM) stroma contains a heterogeneous population of cells, including endothelial
cells, fibroblasts, adipocytes and osteogenic cells. At least two distinct stem cell populations reside in the bone marrow of many mammalian species. They are hematopoietic stem cells (HSCs) and non-hematopoietic stem cells. These non-hematopoietic stem cells, are also termed as multipotent marrow stromal cells or mesenchymal stem cells (MSCs).

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can differentiate into connective skeletal tissue, bone, cartilage, marrow-stroma, and adipocytes. In addition, controversial data suggest that MSCs may give rise to sarcomeric muscle (skeletal and cardiac) (Yu et al., 2007), endothelial cells, and even cells of non-mesodermal origin, such as hepatocytes and neural cells (Miura et al., 2003).

With this wide range of differentiation potential MSCs finds the possibility in engraftment and immunosuppressive effect. Their expansion through culture led to increasing clinical interest in the use of MSCs, through intravenous infusion, in numerous pathologic situations.

During embryonic development, mesenchyme or the embryonic mesoderm contains stem cells that differentiate into virtually all connective tissue phenotypes such as bone, cartilage, bone marrow stroma, interstitial fibrous tissue, skeletal muscle, dense fibrous tissues such as tendons and ligaments, as well as adipose tissue.

MSCs have been isolated from various tissues. The different sources could be umbilical cord blood, chorionic villi of the placenta (Igura et al., 2004), amniotic fluid (Tsai et al., 2004), peripheral blood (Zvaifler et al., 2000), fetal liver (Campagnoli et al., 2001), lung (Noort et al., 2003) and even in exfoliated deciduous teeth (Miura et al., 2003). There are an increasing number of reports describing their presence in adipose tissue (Mitchell et al., 2006) and dental pulp (Gronthos et al., 2001). Various methodologies have been developed to isolate and expand them. It has been noted that stem cells present in adults takes part in normal tissue regeneration in response to disease or injury. Mesenchymal stem cells (MSCs) are one such type of cell that takes part in tissue regeneration. Related to this subject a large amount of recent works have been reported. Some of which is quite promising, suggesting that MSCs can be therapeutically useful in specific settings. These characteristic features of stem cells can be used for specific tissue engineering & organ development.

The promising roles of stem cells have paved way to novel cell-based therapies. For such pervasive and debilitating diseases, scientists must be able to manipulate stem cells so that
they possess the necessary characteristics for successful differentiation, transplantation, and engraftment. To be useful for transplant purposes, stem cells must be reproducibly made to:

- Proliferate extensively and generate sufficient quantities of tissue.
- Differentiate into the desired cell type(s).
- Survive in the recipient after transplant.
- Integrate into the surrounding tissue after transplant.
- Function appropriately for the duration of the recipient's life.
- Avoid harming the recipient in any way.

Also, to avoid the problem of immune rejection, scientists are experimenting with different research strategies to generate tissues that will not be rejected.

The stem cell expansion through culture led to increasing clinical interest in the use of MSCs, through intravenous infusion, in numerous clinical situations. The stem cells were first cultured by growing them over mouse embryonic fibroblast (MEF) feeder cells in medium supplemented with fetal bovine serum (Reubinoff et al., 2000). However the culture condition that include animal derived components such as, cells and serum were not suitable for clinical applications (Wang et al., 2005). Hence there is a pressing need for an alternate to serum. The development of defined, cell and serum free substrate for stem cell culture may pave a way towards the cell replacement therapy and regenerative therapy. Few such serum free media were tried with extracellular matrix components of human origin like laminin (Vuoristo et al., 2009), vitronectin, fibronectin, collagen (Braam et al., 2008) and human serum matrix (Stojkovic et al., 2005).

Knowing these facts, merits and demerits of FBS in animal cell culture media, the need of the hour in terms of 3R Principal (Reduction refinement & replacement) is to reduce or to avoid harvesting FBS from bovine foetuses & to find an alternate to the use of FBS. Hence an attempt was made in this study to investigate the feasibility of using plant protein as an alternate or as supplement of FBS. An attempt was also made to explore cell proliferation under exposure to defatted soy bean (Glycine max) flour in order to determine whether this can be used as a complete or partial replacement for serum. Based on the above facts the aim of the study is to isolate, identify, characterize, expand and maintain multipotent stem cells. To find an alternate source for growth factor to add in tissue culture media in lieu of fetal bovine serum / fetal calf serum.