3. Review of Literature

"Exactness cannot be established in the arguments unless it is first introduced into definitions"

- Jules Henri Poincaré
  (French Mathematician, 1911 Bruce Medal, 1854–1912)

The Cell

All living creatures are made of cells, a small membrane-bounded compartments filled with a concentrated aqueous solution of chemicals. The simplest forms of life are self-contained cells that propagate by dividing into two. Like ourselves, the individual cells that form our bodies can grow, reproduce, process information, respond to stimuli, and carry out an amazing array of chemical reactions. These capabilities define life. Human and other multicellular organisms contain billions or trillions of cells organized into complex structures, but many organisms consist of a single cell. Even simple unicellular organisms exhibit all the hallmark properties of life, representing that the cell is the fundamental unit of life. As the Common Era opens, mankind face a flare-up of new data about the components of cells, what structures they contain, how they communicate and influence each other. Still, an immense amount remains to be learned, on the whole about how information flows through cells and how they decide on the most suitable ways to respond (Freshney, 2010).

Cell Culture

Cell culture has become one of the major tools used in the study of life sciences today. Animal tissue culture is the general term for the removal of cells, tissues or organs from an animal and their subsequent placement into an artificial environment conducive to growth, thus disrupting their normal relationship with their neighbouring cells. This culture process occurs in glass or an artificial environment created (in-vitro).

In-vitro methods are widely used tools to study physiological, biological and pharmacological activities at the cell and tissue level. In addition, in-vitro methods are also becoming increasingly important in the production of biological components, such as production of vaccines, antibodies, hormones and cells themselves for regenerative
medicines. Mammalian cells are generally grown under well-established conditions in incubators, where the temperature is typically kept at 37°C with a controlled humidified gas mixture of 5% CO₂ and 95% O₂ (Freshney, 1994).

**Role and Application of Animal Cell Culture**

Classical animal cell technology has been concerned with the production of viruses, viral vectors, and recombinant proteins for different purposes. However, animal and human cells can also be used for patient destined therapies like artificial organs, tissue engineering and transplantation of cells. This is a rather new field and many human diseases either acquired or inherited will be treated by these kinds of therapies in the future. On one hand, these therapies are based on gene therapy by using viral vectors (Urabe et al., 2002) and on the other hand by the development of artificial organs i.e. use of patient destined small scale reactor systems, employable in a clinical setting and tissue repair / tissue engineering, based on the use of stem cell technology have to be mentioned here. In the context of artificial organs, one of the most important potential applications is the transient replacement of a patient’s damaged liver by an artificial one, thus bridging the time gap between liver failure and the availability of one from a matching donor or the regeneration of the patient liver. Different culture approaches are reviewed by Merten in 2006 (Merten et al., 2006). In the future, tissue repair can be achieved by tissue replacement based on the use of stem cells. For this purpose, adult as well as human embryonic stem cells can be used.

The first mammalian cell culture dates back to 20th century in order to study cell physiology and in later 50’s large scale culturing of viruses in cultures of human foreskin and embryonic tissues was performed which led to the production of polio vaccines (Weller et al., 1949). With this as a lead, rabies virus were cultured for vaccine purposes on the established human diploid fibroblast cells like WI 38 and MRC-5 (Hayflick and Moorhead, 1961; Wiktor et al., 1964). Wiktor et al., (1969) also studied the immunogenicity of rabies vaccines by large scale cultivation, which paved the way for industrial production of inactivated rabies vaccine. In the meantime WI 38 cells were used for the production of rabies vaccine (Nicolas et al., 1978). Later in early 60s BHK-21 cells were established by Capstick et al., (1962) based on which the inactivated food and mouth disease (FMD) vaccine was commercially produced. It was also reported that the production process was scaled up to 2500 liter scale (Radlett et al., 1985).
The Food and Drug Administration (FDA) accepted the use of continuous cell lines for the production of biological products for human welfare (Petricciani, 1995; Ozturk, 2006). This led to the commercial production of Interferon (IFN) from Namalwa cells (Pullen et al., 1985) and the inactivated polio vaccine from Vero cells (Montagnon et al., 1984).

The 80s were marked by the advent of mammalian cells based expression systems of heterologous proteins, recombinant CHO technology, the development of the amplification and selection markers Dihydro-folate-reductase (DHFR) and Glutamine-Synthetase (GS) for Chinese hamster ovary cells (CHO) and the development of recombinant baculovirus / insect cells technology. Then the early 90s were marked by the development of recombinant GS-NS0 technology (by Celltech) leading a few years later to the possibility of producing 1–2 gm/l of monoclonal antibodies in a fed batch process (Bibila et al., 1994). The productivity of mammalian cells cultivated in bioreactors has reached the gram per liter range in a number of cases, a more than 100-fold yield improvement over titers seen for similar processes in the mid-1980s. This increase in volumetric productivity has resulted mainly from improvements in media composition and process control. Today the antibody titers are at an industrial scale of 5 gm/l and more (Wurm, 2004; Birch and Biologics, 2005).

**Cell Culture Media**

Successful growth and maintenance of animal cells *in-vitro* requires a culture condition that mimics *in-vivo* condition. To achieve good experimental reproducibility, the composition of the cell culture medium must be corrected. The simplest medium is the classical Ringer’s solution (Ringer and Buxton, 1887), which was developed as a solution with optimal concentrations of different salts to preserve frog heart muscle tissue. To maintain cells and tissues for longer periods of time, the medium also contain components like nutrients and pH buffering substances. This type of medium was formulated by Harry Eagle, who developed Eagle’s minimal essential medium (Eagle’s MEM or MEM). MEM also contained amino acids, glucose and vitamins (Harry, 1955). A similar basal medium, MEM modified by Dulbecco (Dulbecco’s Modified Eagle’s Medium, DMEM), is still used to maintain primary cell cultures and cell lines.

To keep cells alive for longer periods of time and to evaluate proliferation, migration and differentiation, a basal medium must be supplemented with several factors. Serum, from animals or humans, is most commonly used to maintain and proliferate cells. Fetal bovine serum (FBS) serves most purposes and is the present standard. FBS is a complex mixture of

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Different factors and contains a large number of components, like growth factors, proteins, vitamins, trace elements, hormones, etc., essential for the growth and maintenance of cells.

However, the use of FBS is controversial for a number of reasons. First of all, the collection of serum causes unnecessary suffering for the unborn calf (Van der Valk et al., 2004). Secondly, seasonal and continental variations in the serum composition, produces batch-to-batch variations. This, in turn, causes phenotypical differences in the cell cultures, resulting in variations of the results. Additionally, due to the likelihood of contamination (e.g., BSE), the use of animal products is strongly discouraged for production of new biological medicinal products (Van der Valk et al., 2004; Schiff, 2005). In fact, as much as 20 to 50% of commercial FBS is virus-positive (Even et al., 2006).

Basic Components of Media

Mammalian cells are different from microbes as they are incapable of synthesizing a large number of compounds that are needed for their normal functioning. These mandatory substances have to be supplied in the basal medium. A very complex media have been developed containing a large number of inorganic salts, including trace elements, most of the common L amino acids and number of vitamins. Often nucleotide, specific carbohydrates and lipids have to be included (Bjare, 1992).

Inorganic Salts

The inorganic salts help in retaining the osmotic balance of the cell and help in regulating the membrane potential. The salts are chiefly those of Na⁺, K⁺, Mg²⁺, Ca²⁺, Cl⁻, SO₄²⁻, PO₄³⁻ and HCO₃⁻. Divalent cations, particularly Ca²⁺ are required by some cell adhesion molecules, such as cadherin. Ca²⁺ also acts as an intermediary in signal transduction (Alberts et al., 2002). The concentration of Ca²⁺ in the medium can influence whether cells will proliferate or differentiate. Na⁺, K⁺ and Cl⁻ regulate membrane potential, whereas SO₄²⁻, PO₄³⁻ and HCO₃⁻ play role in providing anion required by the matrix and nutritional precursors for macromolecules, as well as regulators of intracellular charges.

Amino acids

The concentration of the amino acids usually limits the maximum cell concentration of attainable. The amino acid balance may influence cell survival and growth rate. All 13 essential amino acids (Arg, Cys, Gln, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, and Val) are
necessary for culturing mammalian cells and are present in high concentrations i.e. 0.5 to 4mM in DMEM. The seven non-essential amino acids (Ala, Asn, Asp, Glu, Gly, Pro, and Ser) are provided by Ham’s F-12. The requirements for amino acid will vary from one cell type to another. Glutamine is required by most of the cells, although some cell lines will utilize glutamate. Glutamine is also used by the cells as a source of energy and carbon (Butler, 2005).

**Vitamins**

Vitamins are provided by the basal medium. At least seven vitamins were found to be essential for cell growth and proliferation. They are choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, and thiamine (Taub, 1990; Bjare, 1992). B-vitamins are necessary for cell biochemistry, and are also present in DMEM as well as in Ham-F-12. Matsuya and Yamane (1986) noticed vitamin B12 requirement at low cell densities that disappeared at higher cell densities. Vitamin B12 and hypoxanthine had a synergistic effect on stimulating cell growth. A combination of hypoxanthine, thymidine and folic acid had a similar cooperative effect. Different cell lines often show specific demands depending upon cell densities. The lipid constituent choline was demonstrated to have a strong stimulatory effect upon growth of human epidermal keratinocytes in the presence of myo-inositol in a defined culture medium. Choline did not stimulate dermal fibroblasts or epidermal melanocytes (Uchida et al., 2009). Thus, if standard media are insufficient for a particular cell line, it is worthwhile testing additional vitamin supplementation for improved growth.

**Glucose**

Glucose is included in most media as a source of energy. It is metabolized principally by glycolysis to form pyruvate, which may be converted to lactate or acetoacetate and may enter the citric acid cycle and is oxidized to form CO$_2$ and water. The accumulation of lactic acid in the medium, particularly evident in embryonic and transformed cells, implies that the citric acid cycle may not function entirely as it does in in-vivo condition. Recent data have shown that much of its carbon is derived from glutamine rather than glucose. This finding may explain the exceptionally high requirement of glutamine or glutamate in cultured cells (Freshney, 2010).
Cell Culture Media Supplements

Proteins

Proteins are carriers for different low molecular weight components and may facilitate cell adhesion. Bovine serum albumin (BSA) is often used as a lipid carrier. However, BSA is derived from animals and may either be contaminated or may contain impurities (Taub, 1990). Nowadays, recombinant proteins, including albumin, are available for animal component-free cell culture (Arunakumari et al., 2010). Serum albumin is the most common choice as protein supplement and is used in many different media, but other proteins are equally effective for some cells (Chu and Robinson, 2001). The functions of proteins in culture media are still debatable and partly theoretical. Very few studies have demonstrated a specific mechanism for media proteins. There are numerous hints that proteins are needed, e.g. to protect the structural integrity of cell surface structures, to act as carriers for proper nutrients, to help serving growth factors adopt the correct structural formation for the cell, to bind agents that are inhibitory, to act as a scavenger in the medium.

For transformed cells the protein requirement can disappear completely. An early study used a human leukaemia cell line that was maintained in a chemically defined medium with no protein supplement gave twice the growth rate for that in serum-containing medium (Buhl and Regan, 1972). This abnormal behaviour is an expression of an altered genotype.

Normal, non-transformed cells do require at least low concentrations of protein. Presence of serum may be inhibitory in some experimental systems. The concentration of concanavalin A (Con A) required for stimulation of lymphocytes was found to be 10 to 20 times greater in the presence of plasma than in the presence of albumin. Defined serum fractions were found to bind Con A. Con A-activated cells needed certain protecting proteins for continued proliferation. These proteins did not themselves promote growth. Serum albumin was, however, needed when lymphocytes were Con A-activated. Albumin or serum-free media abolished growth. It was proposed that serum albumin promoted growth by giving structural integrity to the cell membrane (Santucci et al., 2000). Polet and Spieker-Polet (1975) found that pepsin abolished the growth promoting activity of albumin in lymphocyte culture.

Gorfien et al., (2009) has provided a replacement medium with casein as supplement for suspension cell culture. Glassy et al., (1988) used casein as a basal protein supplement to
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propagate murine T lymphoma cells in a medium consisting of a 1 : 1 mixture of DMEM : Ham's F12 with addition of insulin, transferrin, testosterone and linoleic acid.

**Lipids**

The role of fatty acids and lipids in cell culture has long been neglected. Lipids serve as energy stores, as structural constituents of cellular membranes, and in transport and signalling systems. Some lipids are available in the basal medium. However, essential fatty acids and ethanolamine are recommended as supplements. Water-soluble supplements are commercially available. Serum albumin is a carrier of fatty acids and lipids. Essential fatty acids are components of several serum-free media formulations (Sekula *et al.*, 2008).

In synthetic media lipids often have to be supplied to the cells as liposomes, consisting of mixtures of different lipids. Guilbert and Iscove (1976) added crude soybean lecithin to a synthetic medium that supported hematopoietic cells. This lecithin contained approximately 60% phosphatide (phosphatidyl choline-'lecithin', phosphatidyl ethanolamine and monophosphatidyl inositol) and 40% triglycerides (Lebkowski *et al.*, 1996). Bettger and Ham (1982) cultured human diploid fibroblasts in a medium with liposomes consisting of soybean lecithin, cholesterol, sphingomyelin, vitamin E and vitamin E-acetate. The lecithin could be replaced by purified phospholipids. Darfler (1990) prepared a similar lipid microemulsion that stimulated growth of Chinese hamster ovary cells, hybridomas, lymphoblasts and keratinocytes.

Many cells have an obligatory lipid requirement, while other grows perfectly well without lipid. The specific demands have to be tested for each particular cell types. Two free fatty acids and a carrier protein were found essential for growth of Con A-stimulated lymphocytes. They are palmitic and oleic acids and they supported growth most efficiently in combination with albumin or β-lactoglobulin (Spieker-Polet and Polet, 1981).

Linoleic acid, dipalmitoyl lecithin and cholesterol stimulated the growth of hybridomas. No synergistic effect of the combined lipids was seen. Cholesterol or cholesterol precursors were found obligatory for mouse myeloma cell lines (Lee, 1983), while Ohmori (1988) demonstrated that cholesterol and low density lipoprotein were essential for the antibody response by murine lymphocytes in RPMI-1640 medium (Moore *et al.*, 1967) with added albumin, insulin, transferrin and cyclodextrin. Lipid stimulation in serum-free medium
was almost as efficient as serum addition. Jung et al., (2011) found an inhibitory effect of serum low density lipoprotein (LDL) upon DNA and protein synthesis in cardiac myocytes compared with serum- and lipid-free media.

**Glutamine**

Glutamine is an essential precursor for the synthesis of proteins and ribonucleotides. It is also important as respiratory fuel for rapidly dividing cells and cells that use glucose inefficiently (Mulukutla et al., 2010). However, glutamine also has its drawbacks: it is unstable in solution. Glutamine breakdown and metabolism result in the production and accumulation of ammonia, which is toxic to cells (Quek et al., 2010), since it is not absorbed by serum proteins in serum-free and/or protein-free media. To overcome these disadvantages, alternatives for the use of glutamine in culture media were developed. Glutamate, for example, can replace glutamine in cell cultures that express sufficient glutamine synthetase activity. A more recent invention is the use of glutamine-containing dipeptides, alanylglutamine and glycyl-glutamine, commercially available under the trade name GLUTAMAX™ (Amable and Butler, 2008). These dipeptides are more stable and heat resistant, which even makes it possible to autoclave the media that contain these molecules. The dipeptides are intra- or extracellularly cleaved by peptidases, thereby releasing glutamine and either alanine or glycine. The availability of glutamine is therefore dependent on the peptidase activity, which results in lower rates of glutamine consumption and ammonia production. GLUTAMAX™ can be substituted for glutamine on a 1:1 M basis.

**Transferrin**

Transferrin is also an essential protein in culture medium where the main action is to transfer iron into the cells (Bjare, 1992). The role of transferrin seems to be multifunctional, and it is present in media for lymphoid cells and in media for non-lymphoid cells (Van Der Valk et al., 2010).

Brock (1981) found a remarkable increase of DNA-synthesis in mitogen-stimulated lymphocytes by transferrin addition. No difference was found in serum-enriched and serum-free media. Transferrins from homologous and heterologous species were equally effective. It was further demonstrated that transferrin released iron to lymphoid cells without being degraded and was again recharged with iron for another transfer cycle. Brock (1984) also
found an obligatory requirement of transferrin for lymphocytes activated by Con A. Iron chelates and mono-ferric transferrin fragments were unable to promote transformation. Transferrin was assumed to serve functions other than a source of iron (Brunner et al., 2010).

A different function of transferrin was noted when it was found to counteract the effect of low-density lipoproteins (LDL) in its action on specific mitogen stimulation of lymphocytes. Another example of the diverse effects of transferrin is the interplay of LDL and transferrin in oxidative enzyme stimulated lymphocytes. Presence of LDL blocked the cell cycle. This block was relieved by addition of transferrin (Scupham et al., 1987). In a transferrin deficient hybridoma culture, iron chelators could partially substitute transferrin in transfer of iron to the cells (Yabe et al., 1986; Hentze et al., 2010).

Hormones

All hormones of mammalian organisms are physiological constituents in blood circulation and are thus present in serum in varying amounts (Lindl and Gstraunthaler, 2008). Supplementation with hormones was therefore a first step in the development of serum-free media (Barnes and Sato, 1980). In a monograph from a Cold Spring Harbour Conference on Cell Proliferation, growth of cells in harmony defined media was discussed. Some of the articles dealt with serum-free conditions (Pardee et al., 1982). During the last decade combinations of hormones have successfully been used for an increasing number of cell lines. Primary cell isolates or diploid cells still are trickiest to get growing under these defined conditions (Telfer et al., 2008).

Insulin has been shown to be obligatory in all serum-free media formulations. Other hormones most widely used in serum-free cell culture are glucocorticoids (dexamethasone and hydrocortisone), triiodothyronine (T3), and hormones that cell-specifically act by increasing intracellular cAMP levels. Insulin is the most common hormone supplement of culture media. It is often combined with other hormones (Hu et al., 2009). Serum depletion of rat hepatoma cell cultures caused a halt in early G1 growth phase. By insulin addition cells re-entered the cell cycle and traversed into S phase. This response was dose dependent (Koontz and Iwahashi, 1981). Campbell et al., (2012) suggested that the inclusion of insulin in culture media could be used as a strategy for increasing the efficiency with which the ESC lines can be derived from cultured embryos. One of the examples is that of Silber et al., (2011), who
replaced serum by insulin and hydrocortisone in Eagles-MEM for the production of hepatocytes spheroids.

**Growth factors**

Growth factors are generally added to the basal medium to increase cell proliferation and to stimulate specific cell functions. Traditionally, growth factors and other supplements are added as bulk in the form of fetal bovine serum (FBS).

Most growth factors are highly cell type specific. Others are of more general use and can also have positive effects on several different cell types. Fibroblast growth factor-2, for example, has a positive effect on the phenotype of chondrocytes cultured in serum-free medium (Mandl *et al.*, 2004). Some cells in culture may release growth factors thereby stimulating their own proliferation and that of other cells (Antunes *et al.*, 2010).

The growth response to insulin and insulin-like growth factor 1 (IGF-1) was studied in myeloid leukaemia cells and lymphoma cells. Leukaemia cells propagated with an 18-fold reduction of cell yield in the absence of insulin compared with cell yield in the presence of insulin. Lymphoma cell growth was not affected by presence/absence of insulin or IGF-1. When measuring the amount of insulin or IGF-I binding receptors on cells, a poor correlation was found between actual insulin or IGF-1 growth response (Sinclair *et al.*, 1988).

The action of IGF-1 upon fibroblasts was also studied under serum-free conditions. Glucocorticoids strongly affected IGF-1 action. It was found that preincubation of fibroblasts in the presence of dexamethasone had a pronounced synergistic effect in IGF-I stimulation (Stone *et al.*, 2012). This synergistic hormone effect was also seen when primary cardiac myocytes were grown for over two weeks in a basal medium enriched with insulin and transferrin. Presence of dexamethasone was needed for optimal contractility (Jung *et al.*, 2011).

Saumande (1991) demonstrated the effect of insulin and fibronectin on the response to Follicle Stimulating Hormone (FSH) of bovine granulosa cells in a serum-free medium. The levels of steroid hormones were affected by the combinations of the supplements employed.

Gregory Hamilton and Ham (1977) found a medium called F12 which was found to be insufficient for growth of Chinese hamster ovary cells under protein-free conditions. Growth
was restored when thyroxine was added. This dependency was shown to be due to contamination by small amounts of selenium, which by itself substituted for the thyroxine preparation. A similar behavior was found for Chinese hamster lung cells that required either methylcellulose or insulin. A common impurity was presumed to be present in both substances.

Ham's media were developed with the goal not to adapt cells to the new serum-free medium but to get immediate continued growth as the media should be fully supplemented and should not require adaptation or spontaneous selection of competent clones. However, this approach included storage of the stock cultures in media with 2% serum to avoid spontaneous transformations of cell cultures. But, even under these conditions, some selection was still observed (Ham, 1965).

Ham (1982) composed a complex lipid-enriched basal medium (MCDB 110) supplemented with epidermal growth factor, insulin, dexamethasone, prostaglandin E1 and F2α, phosphoenolpyruvate, dithiothreitol and glutathione to grow diploid fibroblasts like WI-38 and MRC-5 (Tsao et al., 2005). This medium contains less than 1µg protein per ml and gives rapid serum-free growth. McKeehan (1982) reported a reduced requirement for Ca²⁺, K⁺, Mg²⁺, phosphate ions, and 2-oxocarboxylic acid by supplementation with serum-derived growth factors in human fibroblast cultures.

Transforming growth factor (TGF) isolated from cells transformed by different means induced normal cells to exhibit transformed cell characteristics. TGF could induce fibroblastic cells to reduced serum requirements. Removal of TGF on the other hand did revert the cell phenotype to normal (Zavadil et al., 2004)

Chiang et al., (1985) used the relaxed nutrient requirements of transformed cells as a selective approach in isolating virus- or oncogene-transformed cells. By excluding insulin, epidermal growth factor and high density lipoprotein from the serum-free medium, conditions were created that required cell transformation for growth, demonstrating the link between cell growth factor requirement and oncogenes. A similar approach was used by Shirahata et al., (2005), who developed an experimental model for characterization of oncogenes capable of conferring growth factor independence. In serum-free medium transformed cells with relaxed factor requirements and loss of contact inhibition could be selected. In another similar investigation it was found that transduction of human proto-oncogene, encoding the receptor
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for colony stimulating factor 1, into mouse NIH 3T3 cells could replace the requirement for platelet derived growth factor (PDGF) and for insulin. Coinciding with this relaxed demand, cells acquired a partially transformed phenotype (Roussel and Sherr, 1989). The same factor, PDGF, or a factor released from activated mononuclear phagocytes, was used by Rutherford et al., (1982), to induce replication of fibroblasts in the absence of serum. The capacity of the cells to respond to this factor in culture diminished with time. Replication resumed on addition of serum.

A dose dependent effect of recombinant colony stimulating factor and recombinant interleukin was demonstrated in human megakaryocyte progenitors grown in serum-free media (Xi et al., 2008).

Jackson and Shin (1982) investigated the function of inositol as a growth factor for mammalian cells in culture by fractionating serum and using the inositol-free fraction to test for inositol-requeriment. Different cell lines varied greatly in their requirements for inositol from absolute dependence to total independence. Inositol is built into a membrane phospholipid and has to be synthesized by the non-dependent cells.

**Serum**

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, Hepatitis B and C. 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.
Protein hydrolysates

Protein hydrolysates are used to deliver amino acids and small peptides. These are not essential in cell culture and the effect is somewhat controversial. In fact, some studies report a beneficial effect in cell cultures (Burteau et al., 2003), while other studies demonstrated that protein hydrolysates do not support cell growth and that higher concentrations actually reduce cell growth (Keay, 2004). Protein hydrolysates are chemically not defined and may cause problems in reproducibility and comparability of experiments (Lobo-Alfonso et al., 2010).

Protease Inhibitors

The protease inhibitors that are introduced by the addition of FBS are α1-antitrypsin and α2-macroglobulin (Gstraunthaler et al., 2008). These inhibitors terminate the trypsination process and act beneficially by inhibiting lysosomal peptidases that may occasionally be released during cell turnover. Protease inhibitors thus have a protective effect on cells, but are not essential. When no protease inhibitors are supplied, one should carefully assess the trypsin concentration.

Attachment Factors

Most mammalian cells need a special culture substratum for cell attachment in order to survive and grow in-vitro. The plastic culture dish, that is specifically treated to introduce charge and hydrophilicity into the polystyrol surface, e.g., with poly-L or D-lysine or ornithine, is the most commonly used substrate for cell attachment. Coating the plastic dishes with other substrates like extracellular matrix components (Guilak et al., 2009) or collagenous matrices (Hurskainen et al., 2010) further facilitates the adhesion of anchorage-dependent cells.

Shear force protector

Turbulence in bioreactors and perfusion cultures cause shear stress in cells. Serum protects cells from this shear force (Elias et al., 1995; van der Pol and Tramper, 1998; Chisti, 2000). Pluronic F68 has a similar effect (Palomares et al., 2000), but is not essential for ordinary cell cultures. Leung et al., (2010) had used shear force protectors in agitation culture which induce differentiation of human pluripotent stem cells in microcarrier cultures.
Antibiotics

Addition of antibiotics to the cell culture media facilitates long-term cell line propagation without any contamination. The use of laminar hood and aseptic techniques reduce the addition of antibiotics in the cell culture media. Common antibiotics used in tissue culture are moderately effective in controlling bacterial infections and are listed in Table 3.1 (Perlman, 1979). Indeed, addition of antibiotics also have demerits like

- antibiotic-resistant microorganism may develop
- adverse effects on cell growth and function
- may hide mycoplasma infections
- anti-metabolic effects that can cross-react with mammalian cells
- encourage poor aseptic technique

However, a significant number of bacterial strains are resistant to antibiotics, either naturally or by selection, so the control that they provide is never absolute. Fungal and yeast contaminations are particularly hard to control with antibiotics; they may be held in check, but are seldom eliminated. Hence, wherever possible, the use of antibiotics should be avoided (Kuhlmann, 1995).

pH

Most cell lines grow well at pH 7.4. Although the optimum pH for cell growth varies relatively little among different cell strains, some normal fibroblast line perform best at pH 7.4 to 7.7, and transformed cells may do better at pH 7.0 to 7.4 (Eagle and Piez, 1962). It was reported that epidermal cells could be maintained at pH 5.5 (Freshney, 2010) but this level was not universally adopted. Phenol red is commonly used as an indicator. It is red at pH 7.4 and becomes orange at pH 7.0, yellow at pH 6.5, lemon yellow below pH 6.5, more pink in at pH 7.6 and purple at pH 7.8.

Complete Cell Culture Media

The term complete medium implies a medium that has had all its constituents and supplements added. It is usually made up of a defined medium component, some of the constituents of which, such as glutamine, may be added just before use, and various supplements, such as serum, growth factors, or hormones. Defined media range in complexity
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Table 3.1. Antibiotics used in animal cell culture media

<table>
<thead>
<tr>
<th>No.</th>
<th>Antibiotic</th>
<th>Concentration in µg/ml (Unless otherwise stated)</th>
<th>Activity against</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Working</td>
<td>Cytotoxic</td>
</tr>
<tr>
<td>1.</td>
<td>Amphotericin B</td>
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<tr>
<td>2.</td>
<td>Ampicillin</td>
<td>2.5</td>
<td>--</td>
</tr>
<tr>
<td>3.</td>
<td>Ciprofloxacin</td>
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<td>--</td>
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<td>Erythromycin</td>
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<td>300</td>
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<tr>
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<td>Gentamycin Gentamicin</td>
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<td>&gt;300</td>
</tr>
<tr>
<td>6.</td>
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<td>MRA (ICN)</td>
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<td>Neomycin</td>
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<td>Nystatin</td>
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<td>10,000 U/mL</td>
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</tbody>
</table>
from the relatively simple Eagle’s MEM (Eagle, 1959), which contains essential amino acids, vitamins, and salts, to complex media such as medium 199 (M199) (Morgan et al., 1950), CMRL 1066 (Langdon, 2008), MB 752/1, RPMI 1640 (Moore et al., 1967), and F12 (Ham, 1965).

**Potential problems with the use of Serum**

There are a number of serious disadvantages incurred when serum is used as a supplement in culture medium. These have different impacts depending on the intended use of the cultured cells. In the production of biopharmaceuticals, compliance with rigorous regulatory controls concerning potential contamination by viruses and other adventitious agents is a primary concern as it is the need to purify the final product from contaminating proteins. These concerns may be of limited relevance in research studies where an accurate knowledge of growth factor content may be more critical. The main difficulties encountered using serum are as follows:

*Lack of reproducibility*

Serum batches vary considerably depending on the characteristics of the source animal used, on the feed stuff employed, on the time of year etc. Different batches contain different absolute and relative levels of growth factors. Certain factors may be deficient in some batches while others may be present in excessive levels that would be inhibitory for some cells. In experimental cell biology, it is also important to be aware of the inherent variability of serum which renders it very difficult to study the specific effects of molecules such as growth factor, hormones, cytokines, adhesion molecules or matrix components, all of which are present in serum at undefined and variable levels (Davis, 2002).

The presence of specific antibodies in serum may also profoundly affect the results obtained. This is particularly true in the production of viruses. Antibodies in the serum may result from a natural infection with the relevant virus or a related species or from a prior vaccination of the animals used. The point to be noted is some antibodies may also cross the placenta. Serum also varies depending on the quality and reproducibility of the procedures used for its collection. For instance, the length of the time between collection of blood and removal of cells is critical if the lysis of cells and the release of cell contents are to be kept
low. Sterility of the operation, exclusion of pyrogens and several other process parameters are also critical.

Risk of contamination

Serum represents a major potential route for the introduction of adventitious agents including bacteria, fungi, mycoplasma and viruses into cell culture. This could be disruptive in research, dangerous in pharmaceutical manufactures and regenerative therapy. In order to reduce the risk of contamination, suppliers must apply rigorous health check to animals used, use good manufacturing procedures (GMP) facilities for the collection and processing of serum, apply through quality control testing and ensure severe batch documentation to permit verification of all the procedures (Dedrick, 1997).

As there was an outbreak of bovine spongiform encephalopathy (BSE) in 1993 and it cannot be removed by filtration and there by cannot eliminate the risk of contamination. As further line of defence, regulatory authorities now require that only serum from specific countries of origin, where particular viruses are thought not to occur, can be used in production of pharmaceutical, veterinary and sometimes diagnostic products (Hodgson, 1993). To further reduce the risk of viral contamination, serum can be subjected to virus inactivation with chemicals such as β-propiolactone. Heat inactivation also inactivates some viruses. In general, all of these processes also results in decreased growth promoting activity and increased cost (Coecke et al., 2007).

Availability and cost

Serum is a by-product of the meat industry. As such, its supply depends on the agricultural policies in the different supplying countries. The supply of genuine New Zealand FCS is very limited and this product therefore commands a very high price. Whatever the origin, very significant investment and operating costs are incurred by any manufacturer who produces FCS according to GNP principles (Van Der Valk et al., 2010).

Influence on downstream processing

The presence of serum in tissue culture medium presents particular difficulties when purifying products secreted by cells. At 10% concentration, serum contributes about 4-8mg/ml of protein. While recombinant proteins are expressed at high levels of 10mg/ml. In
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In this situation, efficient purification of the required protein may be difficult and in some cases it may even be impossible to devise an economically acceptable purification process. Monoclonal antibodies may be secreted at higher levels (400 - 600µg/ml) but are particularly difficult to purify from serum containing medium because of the high level of endogenous immunoglobulins.

Downstream processing may account for over 80% of the cost of commercial production of pure proteins and may determine the commercial viability of the whole process. In such circumstances, dependence of the upstream process on serum supplementation can be a critical disadvantage. One approach which is sometime used to reduce this problem is to change to a low serum concentration when cell growth is completed so as to reduce the concentration of serum during the product generation phase of the process (Cartwright and Shah, 1994).

Serum Replacement and Serum Free Media

The efforts to identify all the serum components that are physiologically relevant to maintain proliferation of cells in culture, and the attempts to replace the serum with its defined components, were not successful (Taub, 1990). Since then, several different serum-free formulations have been developed where the media are supplemented with approximately 10 essential components. About 10 to 20% of these strategies appeared to be successful (Pazos et al., 2004).

An attempt to simulate serum composition was the development of Eagle's basal medium for cell activation (Eagle, 1955). Starting with a complex medium, he simplified it by removing various components and lowering concentrations down to a basal level of requirements, 'minimum essential medium' (MEM). Eagle (1955) showed that L-cells and HeLa-cells could propagate at low concentrations of dialyzed serum (1-5%) in a defined medium of amino acids, vitamins, cofactors, carbohydrates and salts. Omission of single component resulted in death of the cells in culture. For L- and HeLa-cells 13 amino acids were essential for proliferation. Only L-amino acids were active. For most amino acids optimal concentrations were below those that are present in serum and significant increases of concentrations were inhibitory. Seven vitamins were found vital for growth and are choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin and thiamine. A number of other
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Essential vitamins were assumed to be present as trace contaminants in the other components and thus considered as nonessential (Yang and Basu, 2012).

Hydrolysates of different cells or tissues, peptones, have been used as supplements in cell culture media for a long time. Many commercial peptones are available by an analytical protocol. But these additives are defined only by name and not literally by composition as their complexity equals to that of serum. Some aspects of cell culture with peptone media have been reviewed by Taylor and Parshad (1977).

Katsuta and Takaoka (1973) published number of papers on this subject during 1960s, which they summarized in a review in 1973. Their complex medium permitted growth of a number of transformed rat cell lines and some tumour cell lines from other species. Most cell lines required three to ten time greater inoculum than for growth in serum media. Some cell lines had an obligatory requirement for inositol. In a second review Katsuta and Takaoka (1976) defined mixtures of what they called 'multipurpose synthetic media' that permitted growth of various tumour cells. Significant for these media were their high amino acid concentrations, two to three times those of standard media.

Eagle and Piez (1962) also stressed the necessity of dense inoculum for continued cell growth in the absence of serum. It was proved that cultured mammalian cells synthesized serine but, if the cell density was too low, the amount of serine released to the medium was insufficient for survival of the cells. A corresponding population-dependent requirement was also described for cystine, asparagine, glutamine, inositol and pyruvate. Quoting Eagle: "The critical population density was that which was able to 'condition' the medium, i.e. to build up a concentration in equilibrium with the maximum effective intracellular level, before the cells died of specific deficiency".

An example of simplified requirements is a mouse cell line (L-M) that was grown continuously in twice the concentration of Eagle's basal medium with methylcellulose as sole supplement. Methylcellulose was considered to simply prevent clumping (Mukavitz Kramer and Carver, 1986).

Ham (1965) composed a defined medium for Chinese hamster ovary cell lines and L-cells. This synthetic medium, F12, contains 47 ingredients which included all the common amino acids and vitamins. Because of its rich composition F12 has become a popular
component in many mixed media. It is often combined with equal amounts of Dulbecco's modified Eagle's medium (DMEM) (Conigrave et al., 2000).

Dulbecco (1970) studied the serum requirements of different transformed cell lines showing the effect of serum depletion upon DNA synthesis and the proportion of cells entering mitosis. For many cells, initiation of DNA-synthesis had a low level dependence on serum concentration, while the capacity to undergo mitosis was strongly dependent on the serum level (Bard and Eladale, 2009).

In order to get the serum requirement experiment was performed by Dimova et al., (2010) who could induce serum-free growth in several cell lines by SV40 (simian virus) infection that released an obligatory serum requirement. The number of cell divisions induced was proportional to the SV40 multiplicity of infection. It was suggested that the SV40 infection induced the required serum growth factor.

The revolutionary work by various scientific teams replacing serum by the addition of selected hormones, promoting growth and differentiation of specific cells, led to the development of a better chemically defined, serum-free media (Taub, 1990; Bjare, 1992; Grillberger et al., 2009; Van Der Valk et al., 2010).

Based on the literatures animal cell culture media without serum or with serum supplement are classified and define as follows:

1) Serum-free media: serum-free media do not require supplementation with serum, but may contain discrete proteins or bulk protein fractions (e.g., animal tissue or plant extracts) and are thus regarded as chemically undefined.

2) Protein-free media: protein-free media do not contain high molecular weight proteins or protein fractions, but may contain peptide fractions (protein hydrolysates), and are thus not chemically defined. Protein-free media facilitate the down-stream processing of recombinant proteins and the isolation of cellular products (e.g., monoclonal antibodies).

3) Animal-derived component-free media: media containing no components of animal or human origin. These media are not necessarily chemically defined (e.g., when they contain bacterial or yeast hydrolysates, or plant extracts).
4) Chemically defined media: chemically defined media do not contain proteins, hydrolysates or any other components of unknown composition. Highly purified hormones or growth factors added can be of either animal or plant origin, or are supplemented as recombinant products.

In the past decade many investigations were carried out to develop serum replacement and serum free cell culture media. More than 100 different serum free media formulations have been developed and are available for ready use (Brunner et al., 2010).

Alternatives to FBS in cell culture

The removal of serum from the cell culture media and/or replacement with other complex biological fluids and extracts initiate diverse variations in the interactive nature of the cell culture that are introduced in a single step.

Jayme et al., (1988) has described various methods to reduce the requirements of FBS in culture media as well as alternative animal serum substitutions. A poorly defined medium supplement (FBS) is replaced by another ill-defined product. This includes the substitution of FBS with newborn or adult calf sera, or the use of sera from other animal species like horse (Bottenstein and Sato, 1979), pig (David, 1966), goat (Brewer et al., 1993; Paranjape, 2004), mouse (Müllbacher et al., 1985), chicken (Nanda et al., 2009) etc.

Other animal derived alternatives are tissue extracts like pituitary extracts, chicken embryo extract, bovine milk fractions, or bovine colostrum (Klagsbrun, 1980; Steimer et al., 1981; Pakkanen and Neutra, 1994; Belford et al., 1995).

Kannan et al., (2009) evaluated Tualang honey as a supplement to fetal bovine serum in cell cultures and found an enhanced cell proliferation corresponding to the decrease in concentrations of honey as indicated by the mitotic index values when the osteoblast cell line was incubated at 37°C for 48 hours. There were no chromosome aberrations in the honey treated CRL1543 cells

Sagirkaya et al., (2004) showed that FCS replaced with synthetic serum supplement in the in-vitro maturation medium is necessary to obtain a higher cleavage rate and development rate to the blastocyst stage of immature bovine oocytes recovered from ovaries obtained from slaughter houses.
Shetty et al., (2007) worked on swine Bone Marrow MSC and cultured in the presence of FBS and human umbilical cord blood serum and found that both of them were morphologically and phenotypically similar. Human umbilical cord blood serum supported the growth of MSC. While no significant differences were observed in the MSC numbers in swine cells cultured in the presence of FBS or CBS, human cells showed a greater proliferation potential in the presence of CBS as compared to FBS.

Meanwhile Kocaoemer et al., (2007) showed that pooled human AB serum and thrombin-activated platelet-rich plasma are alternatives to FCS for adipose tissue MSCs. These human sources were better characterized regarding potential infectious threats, while providing a higher proliferation rate and retaining differentiation capacity and mesenchymal stem cell marker expression throughout long-term culture.

**Commercially available Serum Free Media**

A number of serum-free media have now been marketed. There are two groups of such media. One provides a complete list of all ingredients. These media certainly save much laboratory work and are useful for small scale culture. The other group, with a complex mixture of ingredients, does not furnish exact formulations, which are proprietary information. These media are useless for studies of cellular functions, since conclusions have to be drawn with a 'black box' as part of the experiments. These media may, however, give excellent growth. Hence to meet out the need plant based serum free medium with complete ingredients have been tried (Bjare, 1992; Brunner et al., 2010).

**Plant Based Serum Replacements**

As reported by Kunova et al., (2010) culture conditions that include animal derived cells and serum are not suitable for clinical applications. This has led to another avenue of investigation to the development of plant derived serum replacements that can be produced in high quantity and with easy quality control.

Plant isolates are used in the elaboration of many food products in order to improve their functional and nutritional properties. Protein hydrolysates have the additional advantages of having improved functional properties (Girón-Calle et al., 2008). Peptones of soybean protein, wheat gluten and Chickpea have been used to supplement basal medium as an
additional source of amino acids, oligopeptide, lipids and trace elements in order to increase the cell proliferation (Franěk et al., 2000; Chun et al., 2007; Girón-Calle et al., 2008).

Plant based proteins can be used as an alternate for FBS and may act as a growth factor supplement for *in-vitro* cell culture. In plants, good quantities of proteins are found rich in seeds in the form of storage proteins. The content of proteins present in the seeds varies from 10% to 40% of its dry weight. These storage proteins and amino acids of the seeds play a major role in seed germination. The seeds are rich in primary amino acids like cysteine, methionine and adequate amount of sulphur containing amino acids. These amino acids are essential for the growth of mammalian cells. Based on the content of amino acids in the seeds, storage proteins are classified into albumin, prolamine and globulin (Pazos et al., 2004).

Recently plant derived protein hydrolysates are commercially available (Bacto Soytone) and have proved to be very useful for production of human therapeutics while eliminating the use of animal derived proteins (Franěk et al., 2000). VegetaCell is a hydrolysate of wheat gluten which had shown to promote the survival of hybridoma cells and the production of monoclonal antibodies (Kunova et al., 2010). In addition to culturing of hybridoma cells in plant based serum replacement medium (Franěk et al., 2000), reports are evident for Keratinocytes (Lee et al., 2008; Tudose et al., 2009), Chinese hamster ovary cells (Chun et al., 2007), primary rat hepatocytes (Hamel et al., 2006), BHK-21 cells (Heidemann et al., 2000), insect cells (Kwon et al., 2005), THP-1 and Caco-2 cells (Girón-Calle et al., 2008) grown in plant based serum replacement media.

**Aloe Leaf Gel**

Aloe is a multipurpose medicinal plant and has gained popularity due to its medicinal capacity. The medicinal value of the plant is got from the whole leaf or the gel part of the leaf or the leaf exudates. The most valuable and important part of *Aloe* plant is its leaves as it has the succulent sap. This sap holds high medicinal value. The morphology of the leaf is fleshy. The cuticle of the leaf is covered by a layer of wax. The stomata are deep sunken to avoid transpiration. The mucilage found in the central cells of parenchyma has the medicinal value (Avinash Gandi and Thirunalasundari, 2010).

The central parenchymatous tissue of the aloe leaf has a colourless, tasteless and glutinous gel component. This aloe gel is very sensitive to light and heat, therefore it is removed from the leaf mechanically and preserved, buffered and stabilised immediately. This
instability of the gel may explain some of the conflicting study results. This gel from *Aloe vera* is used in commercial therapeutics and cosmetics preparation (Hamman, 2008).

Polysaccharides like glucogalactomannan, glucose, mannose, arabinose and galactose, the major component of gel provides to aloes glutinous nature. The biological activity of the gel is due to the presence of glycoproteins. Further to polysaccharides and nitrogenous compounds *Aloe vera* had been reported to have non polar lipids, sterols, saponins, terpenoids and many more compounds. Seventeen amino acids has been reported in aloe gel of which arginine is higher followed by glutamine, alanine and histidine. Apart from these *A. vera* gel is reported with aspartic acid, glutamate and serine (Kodym, 1991).

Acemannan, a mucopolysaccharide from the leaves of aloe has been reported to stimulate the immune system directly. This compound is responsible for the action of lymphocytes against alloantigen. There will be an increase of monocyte and macrophage. Acemannan activates macrophages to produce nitric oxide and enhances phagocytosis (Lee et al., 2001; Im et al., 2010).

Aloe leaf gel directly or indirectly has significant bone marrow stimulating properties. Thus the subcutaneous administration of aloe leaf gel significantly increases splenic and peripheral blood cellularity, as well as hematopoietic progenitors in the spleen and bone marrow, as determined by an interleukin-3-responsive colony-forming unit culture assay and a high-proliferative-potential colony-forming-cell (HPP-CFC) assay, a measure of primitive hematopoietic precursors in myelo-suppressed mice (Lee, 2006).

**Chickpea seed**

Chickpea (*Cicer arietinum*) seeds, a member of the Fabaceae family, are an important food. Chickpeas are low-fat legume seeds. Newman *et al.* (1987) found similar protein ratio values of about 2.8 for three chickpea varieties and protein digestibility ranging from 79 to 88%. These results show that chickpea protein quality is equivalent to that of soybean meal.

Del-Angel and Sotelo (1982) compared the nutritive value of mixtures of chickpeas with wheat, triticale, and normal and opaque-2 corn. The results showed that, though the protein contents of the normal and genetically improved opaque-2 corn were similar, the lysine, tryptophan, sulphur amino acid, and leucine contents of the opaque-2 corn and chickpea combinations were better balanced than those prepared with normal corn;
nutritionally optimum combinations consisted of triticale flour plus hard-endosperm opaque-2 corn; (c) the calculated scores based on amino acid composition correlated well with protein ratio values based on rat feeding studies and the use of genetically improved cereal-chickpea mixtures maintains high protein value and promotes desirable baking and other properties of cereals.

Combe et al., (1991) compared the utilization of amino acids from chickpeas, fava beans, and lentils. Methionine was not fully available to rats from any of the three legumes; threonine was not fully available from chickpeas; and arginine and lysine were not fully available from fava beans.

Purified legume storage proteins (chickpea 11S and 7S globulins, faba bean globulins, and lupin globulins) and casein (casein) were subjected to an in-vitro enzyme (pepsin + pancreatin) digestion process. Protein digests were then used in a bicameral Caco-2 cell culture system to determine amino acid transport across the cell monolayer. With digests from legume proteins, absolute amounts of aspartate, glycine, and arginine transported were higher than those found in digested casein, whereas amounts of glutamate, proline, tyrosine, valine, and lysine were lower. However, proportions of amino acids in the basolateral chamber as compared with amounts added in the apical chamber were lower than casein controls for all amino acids except cystine. Results confirm previous in-vivo observations that amino acids from legume proteins are probably absorbed at rates different from those in other proteins of animal origin such as casein (Rubio and Seiquer, 2002).

**Soybean seed**

Soybean (*Glycine max*) seed is a legume of the family Fabaceae. It is an economic source of basic nutrition and can combat the protein needs. Besides, being rich in nutraceutical components like isoflavones, tocopherols and lecithin, use of soybean in daily diet can reduce the risk of numerous killer diseases viz. breast cancer, diabetes, cardiovascular diseases, osteoporosis. Despite these health-promoting virtues, of the total soybean produced in the country merely 5% is tapped for food uses. Apart from an organ of propagation it is a major source of dietary protein, as its protein content of approximately 40% to 50% of the dry weight. The composition and nutrition of soybeans have been studied extensively (Fukushima, 1991).
Istfan et al., (1983a) compared the nutritional value of a soy protein concentrate with a milk protein in young adult men using a 10 day nitrogen balance method. They found that soy protein supported nitrogen equilibrium as well as the animal protein. The mean daily intake of soy concentrate sufficient for nitrogen balance was 95 mg of N/kg. These studies imply that since soy protein is nutritionally equivalent to animal proteins such as egg, milk, fish, and beef, foods containing soy protein merit wider use in human nutrition. Istfan et al., (1983b) also demonstrated that a well-processed soy concentrate can serve as a sole source of nitrogen and essential amino acids for long-term maintenance in adult humans.

Amino acid patterns by themselves may be insufficient to predict utilization of a protein. In soybeans, anti-nutritional factors such as inhibitors of digestive enzymes and hemagglutinins, as well as poor digestibility are all reported to lower nutritional value. Heat improved the nutritional quality of the product. Adverse effects following short- and long-term ingestion of raw soybean meal by mammals and birds have been attributed to the presence of soybean protease inhibitors and lectins. To minimize possible human health hazards and to improve the nutritional quality of soy foods, inhibitors are generally inactivated by heat treatment during food processing or are removed by fractionation. Most commercially heated flours retain 5 to 20% of the original trypsin and chymotrypsin inhibitor activity. The more protracted heating required to destroy all inhibitor activity would damage the nutritive value of soy proteins. Friedman and colleagues successfully developed improved ways to inactivate soybean inhibitors through disulphide interchange (Friedman, 1996).

Screening of several accessions from the USDA Soybean Germplasm Collection showed variation in the content of trypsin inhibitor, sulphur amino acids and lectins, indicating that further screening studies could lead to the discovery of soybeans which yield flour that is safe and nutritious with minimal heating (Domagalski et al., 1992). Soybean seed serves to be a store of amino acids for their germination. The proteins are called as storage proteins. The major storage proteins include albumins and globulins (Wolf, 1977).

The albumin fraction of soybean seed is reported to contain 8% to 20% of the extractable soybean protein. Based on the sedimentation coefficient it is defined as 2S albumins (Youle and Huang, 1981). This fraction has an average molecular weight of 26,000 Da which is composed of a number of proteins with variety of molecular weight. The major
amino acids reported were cysteine, leucine, serine, arginine and isoleucine (Ibragimov et al., 2011).

The globulins are the most widely distributed group of storage protein. They are divided into two groups based on the sedimentation coefficients, 7S vicilin-type globulins and the 11S legumin-type globulins. They both have nutritional significances in that they are deficient in cysteine and methionine, although 11S globulin generally contains slightly higher level of amino acids (Wolf, 1977). The 7S globulins are also called as conglycinin. This protein makes up to 85% of the fraction. Its molecular weight ranges from 140 kDa to 170 kDa. 7S globulins consist of three subunit proteins labelled as α, α’ and β. The α, α’ subunits have an molecular weight of 57 kDa and β subunit has a molecular weight of 42 kDa.

The 11S globulins are also called as glycinin. The 11S fraction comprises of 30% to 50% of the soluble soy protein. The reported molecular weight ranges from 320 kDa to 360 kDa. Based on the isoelectric points 11S glycinin is divided into three acidic and three basic subunits which contain leucine, isoleucine and phenylalanine (Riblett et al., 2001).

Michl and Spurná (1974) have reported that α – globulin has a growth promoting property and this protein induces mitotic activity in mammalian cells in-vitro. He also demonstrated the growth promoting effect both in primary culture and established cell lines.

From the reports of Lovati et al., (1996) 7S globulin from soybean is ready metabolised in human cell culture by a specific uptake and is degraded for its growth. By metabolism of the 7S globulin the human skin fibroblast enhanced expression of LDL receptors were also noted.

The subcellular localization of soybean 7S globulin in HepG2 cells tends to be the key role of α’ subunit on the cell cholesterol homeostasis. This revealed a potentially interesting association of soybean 7S globulin with proteins, such as thioredoxin 1 and cyclophilin B that are involved in cell protection against oxidative stress (Manzoni et al., 2003).

With the known facts and preliminary results, soybean seed was found to be a good source for protein and is taken up by the cells in-vitro for their metabolism and also supported cell proliferation. Hence soybean seed was selected to achieve the aim of this study.
As the cells used in regeneration therapy must be grown under good manufacturing conditions and as culture conditions that include FBS were not suitable for clinical applications (Stojkovic et al., 2005), in this study soybean seed protein was used for the generation of clinical grade stem cell for regeneration therapy.

**Stem Cells**

Stem cells are unspecialized cells in the human body that are capable of becoming specialized cells, each with new specialized cell functions. That is one cell type stems from the other and hence the term “stem cell”. Basically, a stem cell remains uncommitted until it receives a signal to develop into a specialized cell. Stem cells have the remarkable properties of developing into a variety of cell types in the human body. They serve as a repair system by being able to divide without limit to replenish other cells. When stem cells divide, each new cell has the potential to either remain as a stem cell or become another cell type with new special functions. The classical definition of a stem cell is that it possesses three properties, 1) Self-renewal - the ability to go through numerous cycles of cell division while maintaining the undifferentiated state. 2) Potency - the capacity to differentiate into specialized cell types. In the strictest sense, stem cells are to be either totipotent or pluripotent - to be able to give rise to any matured cell type. Multipotent or unipotent progenitor cells are sometimes referred to as stem cells. 3) Homing - whenever the stem cells are given in-vivo they have a tendency to get along with that tissue or organ accordingly.

Another essential property of stem cells is the flexibility in the use of their functional potentials. The stem cells might particularly be characterized by their ability to respond to actual needs of the system. For this the cells require communication among each other and with their microenvironment. Some of the adult stem cells possess the property of transdifferentiation or plasticity. Transdifferentiation is the ability of the cell to transform from one cell lineage to another completely different cell lineage. However, transdifferentiation is not a unique property of all stem cells; it is found that some cells such as pancreatic cells can be converted to hepatocytes and vice versa (Magnus et al., 2008).

On the basis of source or origin, stem cells are as follows:
Embryonic Stem Cells

These cells are pluripotent stem cells that have the potential to differentiate into any cell in the body except the placenta. Pluripotent stem cells have the ability to differentiate into cells of all the three germ layers namely ectoderm, mesoderm and endoderm (Bongso and Lee, 2005). Embryonic stem cells can be derived from a very early stage in human development. They are derived from inner cell mass of the blastocyst stage embryos (Lanza et al., 2004). From embryo, three kinds of mammalian pluripotent stem cell types have been described: embryonic stem cells; embryonic germ cells derived from primordial germ cells, and embryonal carcinoma cells. ESCs are derived from 4 to 5 day old embryos. At this stage, the embryos are spherical and are known as blastocysts (Nielsen, 2008). Each blastocyst consists of 50 to 150 cells and includes three structures: an outer layer of cells, a fluid-filled cavity, and a group of about 30 pluripotent cells at one end of the cavity, called the inner cell mass (Thomson et al., 1998).

Adult Stem Cells

Adult stem cells are undifferentiated cells, found throughout the body after embryonic development. These cells play a major role in replenishment of dying cells and regeneration of damaged tissues. They are also known as somatic stem cells.

The bone marrow contains two types of stem cells. One population is called Hematopoietic stem cell (HSC) that differentiates into all the blood cells in the body. The other population of adult stem cells is called the bone marrow stromal cells that differentiate into bone, cartilage, fat and connective tissue. These bone marrow stromal cells are called as Mesenchymal stem cells (Turksen, 2004).

An adult stem cell undergoes replication by mitosis to produce two daughter cells, one daughter cell which differentiates into a cell which has characteristic morphologies and specialized functions and another daughter cell that retains the property of mother cell of long term cell renewal (Prentice, 2003).

Although the exact source of these adult stem cells is unclear, ideas have been proposed to explain their origin (Table 3.2). They are embryonic cells that were set aside when the tissue first developed. They may be part of a migrating group of embryonic cells
An attempt to use Soybean protein as an alternate for Fetal Bovine Serum in animal cell culture

Table 3.2. Stem cells seen in adult organs and tissues

<table>
<thead>
<tr>
<th>No.</th>
<th>Organs</th>
<th>Stem cell origin / nature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Brain</td>
<td>Stem cells here can become asetrocytes, oligodendrocytes, and neurons. Some scientists report that they may form certain blood cells.</td>
</tr>
<tr>
<td>2.</td>
<td>Eye</td>
<td>Stem cells have been isolated in the cornea and retina.</td>
</tr>
<tr>
<td>3.</td>
<td>Teeth</td>
<td>Found in the dental pulp of the teeth.</td>
</tr>
<tr>
<td>4.</td>
<td>Bone Marrow</td>
<td>These cells give rise to blood cells and the cells that become bone and cartilage. Blood cell precursors are called hematopoietic stem cells, and bone and cartilage cells are called stromal cells.</td>
</tr>
<tr>
<td>5.</td>
<td>Skin</td>
<td>The largest organ in the body has stem cells that are related to the epidermis, epithelium, and hair follicles, which appear to be associated with repair and replacement.</td>
</tr>
<tr>
<td>6.</td>
<td>Endothelium</td>
<td>Cells in the lining of the organs differentiate into blood vessels, arteries, veins, capillaries and into the muscle of the heart.</td>
</tr>
<tr>
<td>7.</td>
<td>Skeletal System</td>
<td>Bone marrow appears to be a promising place which harvests stem cells. These cells may be used during bouts of exercise or during the repair of injury.</td>
</tr>
<tr>
<td>8.</td>
<td>Digestive System</td>
<td>Stem cells are known to repair problems and dysfunction in the lining of the intestine.</td>
</tr>
<tr>
<td>9.</td>
<td>Pancreas</td>
<td>Although not yet proven, stem cells are believed to exist in the pancreas.</td>
</tr>
<tr>
<td>10.</td>
<td>Liver</td>
<td>Although not yet proven, stem cells are believed to work to repair damage to the liver.</td>
</tr>
</tbody>
</table>
that became part of the organs or tissues during early divisions. They may have developed after embryonic formation in some process of differentiation (Young and Black, 2003).

**Mesenchymal 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) (Karp and Leng Teo, 2009).

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., 2008), endothelial cells, and even cells of nonmesodermal origin, such as hepatocytes and neural cells (Cordeiro et al., 2008).

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 either intravenous infusion or site-directed administration, 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 (Castrechini et al., 2010; Abumaree et al., 2012), amniotic fluid (Carraro et al., 2008), peripheral blood (Bensinger et al., 2009), fetal liver (Chou and Lodish, 2010), lung (Kajstura et al., 2011), and even in exfoliated deciduous teeth (Wang et al., 2012). There are an increasing number of reports describing their presence in adipose tissue (Cawthorn et al., 2012) and dental pulp (Akiyama et al., 2012).
A standard set of criteria have been formulated to identify MSCs (Keating, 2012). First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, phenotypically, MSCs express a number of markers, none of which, unfortunately, are specific to MSCs. It is generally agreed that adult human MSCs do not express the hematopoietic markers CD45, CD34, CD14, or CD11. They also do not express the co-stimulatory molecules CD80, CD86, or CD40 or the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule [PECAM]-1), CD18 (leukocyte function-associated antigen-1 [LFA-1]), or CD56 (neuronal cell adhesion molecule-1), but they can express CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), CD71, and Stro-1 as well as the adhesion molecules CD106 (vascular cell adhesion molecule [VCAM]-1), CD166 (activated leukocyte cell adhesion molecule [ALCAM]), intercellular adhesion molecule (ICAM)-1, and CD29. Third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts in-vitro (Mathiasen et al., 2012).

**Adipose Tissue Derived Stem Cells**

**Adipose Tissue**

Adipose tissue is specialized loose connective tissue that is derived from lipoblasts. It is comprised of adipocytes as well as many additional cell types which are not highly visible in histological section. Unlike other organs, adipose tissue is distributed throughout the body in a variety of locations (Fantuzzi, 2005; Trujillo and Scherer, 2006).

Adipose tissue is a complex, essential, and highly active metabolic and endocrine organ. Besides adipocytes, adipose tissue contains connective tissue matrix, nerve tissue, stromal vascular cells, and immune cells. Together these components function as an integrated unit. Adipose tissue not only responds to afferent signals from traditional hormone systems and the central nervous system but also expresses and secretes factors with important endocrine functions. Adipose tissue is also a major site for metabolism of sex steroids and glucocorticoids (Galic et al., 2010).

The adipocyte is unique among cells in that one organelle, the lipid droplet, encompasses greater than 95% of the entire cell body. This lipid droplet serves as a storage vessel for triglycerides that can be released through lipolysis and added to by the process of
triglyceride synthesis. The adipocyte is traditionally viewed as a cell that is primarily involved in energy storage (Trujillo and Scherer, 2006).

In mammals adipose tissue is found in two different forms: White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT).

**White Adipose Tissue**

The white adipose tissue is the predominant type of fatty tissue in humans and serves three functions: heat insulation, mechanical cushion, and most importantly, a source of energy. They consist of fat cells with a single large lipid inclusion occurring singly or in groups. Subcutaneous adipose tissue, found directly below the skin, is an especially important heat insulator in the body, because it conducts heat only one third as readily as other tissues (Trayhurn and Beattie, 2001).

**Brown Adipose Tissue**

Brown adipose tissue derives its colour from rich vascularization and densely packed mitochondria. It is found in various locations, depending upon the species and/or age of the animal. Its sole purpose is heat generation in mammals. Lipid accumulates in each of the fat cells as many individual droplets of varying sizes, (a multilocular pattern) (Virtanen et al., 2009).

Much of the work conducted on adult stem cells has focused on mesenchymal stem cells (MSCs) found within the bone marrow stroma. MSCs differentiate into adipocytes, chondrocytes, osteoblasts and myoblasts *in-vitro* (Kolf et al., 2007) making these stem cells promising candidates for mesodermal defect repair and disease management. However, the clinical use of MSCs has presented problems, including pain, morbidity, and low cell number upon harvest. This has led many researchers to investigate alternate sources for MSCs.

Like marrow, the adipose tissue is a mesodermally derived organ that contains a stromal vascular cell population containing nonadipocytes such as preadipocytes, endothelial cells, pericytes, monocytes, macrophages and stem cells. A more homogenous population of multipotent stem cells emerges in culture under conditions supportive of MSC growth.

This population of stem cells is called as Adipose Derived Stem Cells (ADSCs). It is found that ADSCs are predominantly found in white adipose tissue than in brown adipose
tissue. The ADSCs must show >95% positivity for expression of cell-surface antigens CD29, CD44, CD73, CD90, CD105, and CD166 (Lin et al., 2010).

Adipose tissue is derived from the mesenchyme and contains a supportive stroma that is easily isolated. The stem cell population in the stroma is in significant numbers and exhibits stable growth and proliferation kinetics in culture. Subcutaneous adipose depots are accessible, abundant, and replenishable, thereby providing a potential adult stem cell reservoir for each individual (Mizuno, 2009).

Adipose tissue is a rich source of stem cells, as the frequency of stem cells within adipose tissue range from 1:100 to 1:1500 cells, which far exceeds the frequency of MSCs in bone marrow, which is 1:105 (Zuk et al., 2002).

A tri-potential differentiation capacity i.e. osteogenic, adipogenic and chondrogenic differentiation capacity was observed for most stem cells derived from adipose tissue when compared to those derived from the bone marrow or from the umbilical cord blood (Gimble and Guilak, 2003).

The study and culturing of human stem cells is one of the most rapidly growing areas of cell biology and regenerative medicine. Recent publications have shown that adult stem cells be cultured in the laboratory to produce cultures with the characteristics of particular tissues. This opens the possibilities of using cultured cells to repair tissues with functions impaired by damage of ageing. Applications may include culture of dopamine producing neurons for implantation into the brains of patients with Parkinson’s disease or pancreatic insulin producing cells for diabetes patients. Cultured ADSC are already widely used for surgical repair and also they are also potential host cells for gene therapy (Kocaoemer et al., 2007).

Considerable efforts are directed toward developing FBS-free media and serum-free alternatives for clinical use of human MSC. The growth of MSC in serum free media can be used in regenerative therapy.