1.0

INTRODUCTION
Erythropoietin (EPO) is a glycoprotein which stimulates erythrocyte formation by bone marrow progenitor cells. EPO has significantly improved treatment of anemic patients, by increasing hemoglobin levels and reducing the need for blood transfusion and the quality of life of cancer patients is thus improved and several studies highlight the beneficial role of EPO on the clinical outcome. Some clinical data and a pre-clinical background suggest a detrimental role of EPO in cancer by a possible stimulation of tumor growth. However, there is a need of more clinical trials in order to assess the effects of EPO on tumors and their treatment (Morgan and Maurice, 2007).

EPO is the principal and key regulator of erythrocyte production in mammalian species. In adults, EPO is mainly produced in the kidney and interacts with a specific receptor on responsive precursor cells in the spleen and bone marrow, preferably erythrocyte progenitors. Later reports suggest that megakaryocytes are also targets for this hormone (Erslev, 1953 and Krantz, 1991).

EPO is a cytokine that stimulates erythropoiesis, the formation of red blood cells. The mature EPO protein consists of 166 amino acids. Its predicted tertiary structure is an anti-parallel bundle of four α-helices. In addition to tissue-specific control, regulation of EPO production occurs mainly at the transcriptional level, and the production of EPO can be induced under anemic and hypoxic conditions (Krantz, 1991).

The expansion and maturation of committed precursor cells in vivo is accomplished in conjunction with other cytokine components, such as IL-3, GM-CSF and the stem cell factor (Nocka et al., 1990). Several studies, using immortalized erythroblasts or erythroleukemia cell lines demonstrate that EPO can induce signals leading to both proliferation and differentiation (Steinlein et al., 1994).

In haemopoietic tissues, EPO is an essential growth factor for erythrocytic progenitors and its survival and EPO exerts its activity by binding to specific cell surface receptors on erythroid progenitor cells. EPO is the primary cause of anemia associated with chronic renal failure (Jelkmann, 1986). EPO promotes the differentiation and proliferation of erythroid cells leading to a significant increase in the number of reticulocytes in the blood, which consequently increases the blood’s hematocrit and haemoglobin concentration (Hitomi et al., 1988).
There are several erythropoiesis stimulating agents in clinical use and in development that either act as ligands for the cell surface receptors of EPO or promote EPO production, which stimulates erythropoiesis (Neeraj and Joseph, 2008). EPO is produced mainly by the kidneys (primarily by cells of the peri-tubular capillary endothelium) in human adults (Jacobson et al., 1957 and Jelkmann, 1986). EPO is also produced locally by other tissues in a paracrine-autocrine manner, in addition to the kidney (Klein et al., 1989).

Clinical investigations reported that, anemia caused due to decreased level of EPO often results in renal failure (Coleman and Brines, 2004) and erythropoiesis usually fails because of a blunting of the kidney-erythropoietin-bone marrow axis during severe illness. In this condition, it was clinically proven that the administration of pharmacological amounts of recombinant human erythropoietin (rhEPO) reduces the need for blood transfusions significantly (http://www.caspases.org/showabstract.php?pmid=15469595). In severe anemic condition, due to insufficient oxygen supply to the tissue, plasma levels of EPO can increase by one hundred fold while its concentration can plummet as a result of renal dysfunction.

An increase in the understanding of the process of erythropoiesis raises some interesting questions about the pathophysiology, diagnosis and treatment of anemia and erythrocytosis. The mechanisms that are underlying the development of many of the erythrocytoses, previously characterized as idiopathic, have been elucidated leading to an increased understanding of oxygen homeostasis. Characterization of anemia and erythrocytosis in relation to serum erythropoietin levels can be a useful addition to clinical diagnostic criteria and provide a rationale for treatment with erythropoiesis stimulating agents (ESAs) (Vivien et al., 2007).

Erythropoietin (EPO), the major hormone that regulates erythropoiesis, is produced mainly in the adult kidney in response to hypoxia and thus considered a major treatment for various types of anemia. The past decade has revealed extra-hematopoietic sites of EPO production along with an abundance of EPO receptors in various tissues and cell lines, suggesting that this hormone may have pleiotropic activities. The unexpected discovery that EPO and its receptor are expressed also in cells of the central
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nervous system has opened new avenues for research regarding the biological effects of EPO in the brain (Sara et al., 2006).

The red blood cell was the first identified blood cell type, and its oxygen-carrying function by hemoglobin was observed by Hoppe-Seyler in the 19th century. However, the regulation of red blood cell formation, or erythropoiesis, remained unknown until 1906 when Carnot and Deflandre first introduced the idea of a humoral mediator for erythropoiesis. Almost 50 years later, Erslev and Reissmann (1953) formally tested this hypothesis experimentally by injecting serum from anemic rabbits into normal rabbits and noting a significant increase of red blood cells in the recipient animals. They further proved this hypothesis by using a parabiotic model in rats to demonstrate a circulating erythropoiesis-stimulating factor, now known as erythropoietin (EPO) (http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B782W-4B0D1X2-2V&_user=10&_orig=full&_sortdate=2003&_rdoc=1&_doctype=C000050221&_version=1&_origin=article&_acct=C000050221&_version=1&_origin=article&_acct=C000050221&_version=1&_urlVersion=0&md5=5756114f19b88c0b40b98b8f1481f4f8).

Erythropoiesis, the development of red blood cells, is a tightly regulated process for maintaining sufficient oxygen delivery to tissue. Oxygen delivery is proportional to the concentration of oxyhemoglobin and cardiac output (http://dwb.unl.edu/teacher/nsf/cl10/cl10links/www.emory.edu/INT_MED_REV/Atlanta/paper/paper.htm). Peritubular interstitial cells in the kidney sense the tissue oxygenation level and control erythropoiesis in the bone marrow by producing erythropoietin (EPO) when more oxygenation is needed. Erythropoietin is the principal hormone that regulates erythropoiesis and its transcription is mediated by hypoxia inducible factor-1 (HIF-1) (Joachim, 2004).

Binding of EPO to its receptors (EPO-R) stimulates erythroid cell division and proliferation and inhibits erythroid progenitor apoptosis. In addition to EPO, other hormones/cytokines have been shown to influence erythropoiesis such as GATA-1, Bcl-XL, FOG-1, and PU-1. In human, the process of erythropoiesis starts initially in the yolk sac, then switches to the fetal liver in the second gestational month. After birth, erythropoiesis occurs in the bone marrow. In cases in which the demand for increased erythropoiesis is required, the process can occur extramedullary in the liver and spleen.
This is the situation early in life, due to the lack of marrow reserve space. This is not the case in adults since they have more abundant marrow space, which is sufficient for increases in erythropoiesis. The significant increase in the knowledge during the last half century about the hormone erythropoietin is reviewed and the description of these events has been separated into two parts. The vigorous changes in response to tissue oxygenation in the kidneys changes the rate of erythropoietin production and the changes in erythropoietin concentrations act on erythroid progenitor cells, resulting in prompt changes in rates of erythrocyte production. These two aspects of EPO biology provide an explanation for the tight physiological regulation of the numbers of circulating erythrocytes and, in a more general manner, provide a model for the control of the numbers of other specific blood cells by their respective hematopoietic growth factors (Mark, 2005).

EPO is the first known hematopoietic growth factor. It was described by Paul Carnot and Deflandre in 1906 and regulation by hypoxia and renal production were demonstrated in 1957. Goldwasser purified the human hormone after twenty years and the EPO gene was cloned in 1985. This permitted confirmation of the peptide structure and large scale production by introducing the gene into mammalian cells, and the first therapeutic use in hemodialysed patients. EPO is a glycoprotein consisting of a polypeptide core of 165 amino acids and the molecule is heavily glycosylated, with a molecular weight of 34 kDa. Glycosylation is necessary for the in vivo activity of the hormone. EPO acts on the late progenitors of the erythroblastic pathway. EPO is synthesized in the endothelial peritubular cells of the kidney. EPO dosage can be performed by in vivo techniques in polycythemic mice and in vitro. Radioimmunoassay has been performed using rhEPO and potent antibodies. EPO has been used successfully in the treatment of anemia resulting from renal insufficiency (Casadevall et al., 1989).

Kidney is the main organ in the adult human for the production and release of EPO and it stimulates erythropoiesis by increasing the proliferation, differentiation and
maturation of the erythroid precursors. Significant efforts were made in the purification, molecular encoding and description of the EPO gene during the last decades and this led to an incredible increase in the understanding of the EPO-feedback-regulation loop at a molecular level, especially the oxygen-dependent EPO gene expression, a key function in the regulation loop. Studies in humans, however, at a systemic level are still very scanty and therefore, it is required to report a review on the main recent investigations on EPO production and release in humans under different environmental and experimental conditions, including: (i) studies on EPO circadian, monthly and even annual variations (ii) studies in connection with short, medium and long term exercise at sea level (iii) studies performed at moderate and high altitude (Gunga et al., 2007).

Erythropoietin is both a hormone and a growth factor. It fits the classical definition of a hormone being made in one organ, the mammalian kidney, being secreted into the bloodstream, and acting at another site, the blood-forming system, i.e., the bone marrow in adults. As a growth factor, it is required for red blood cell formation from more primitive cells of the hematopoietic system. In adult human beings, it regulates normal erythropoiesis, stimulating the production of approximately 2.5 million red cells per second (http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B782W-4B0D1X2-2W&_user=10&_origUdi=B6VM6-4G4X02N-CN&_fmt=high&_coverDate=11%2F12%2F2003&_rdoc=1&_orig=article&_origin=article&_zone=related_ref&_acct=C000050221&_version=1&_urlVersion=0&_userid=10&md5=51c6a57e31b5320b710ee3c8f0507b06).

**Physiology**

Circulating EPO, which is required for red blood cell production, is primarily produced in the kidney, but also in various other tissues (including the nervous system and the heart) and mediates its effects by binding to its specific cell surface receptor (EPO-R). The EPO-receptor (EPO-R) is also expressed in many non-haematopoietic cell types, including neurons and cardiomyocytes and it has been shown that EPO acts as a potent cell protective and trophic factor (http://www.rofar.org/rainbow/portal/alias_rofar/lang_en-US/tabID_3353/DesktopDefault.aspx).

Oxygen is essential for life. The oxygen consumption of the human body varies based on an individual's metabolic demand and can be limited by the environmental
oxygen concentration. Early observations provided links between symptoms such as fatigue, shortness of breath, and cardiac palpitations and either low oxygen tension or anemia. Further studies showed that low oxygen tension resulted in an increased number of red blood cells, the cells responsible for the delivery of oxygen to systemic organs and tissues (http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B782W-4B0D1X 2-2V&_user=10&_origUdi=B6VM6-4G4X02N-CN&_fmt=high&_coverDate=11%2F1 2%2F2003&_rdoc=1&_orig=article&_origin=article&_zone=related_ref&_acct=C00 0050221&_version=1&_urlVersion=0&_userid=10&md5=5756114f19b88c0b40b9b8fb 148194f8).

Erythropoietin stimulates the synthesis of hemoglobin by acting as the main regulator of the production of red blood cells and it also functions in the recruitment and differentiation of erythroid progenitor cells and aids in their maintenance and survival. The ready availability of recombinant human erythropoietin, in the last 15 years, has permitted the clinical investigation and application of this hormone to the treatment of anemia in various patient populations (Bieber, 2001).

EPO neuroprotective activity is not teleologically distinct from the erythropoietic one (Marti et al., 1996; Masuda et al., 1994 and Marti et al., 1997) and EPO production by hypoxia occurs through hypoxia-inducible factor 1 (HIF-1), which also induces expression of several glycolytic enzymes to reorient energy metabolism toward favoring survival during hypoxia (Semenza, 2000). Increased production of EPO by kidney represents the main adaptive mechanism to hypoxia by the organism through augmenting the number of erythrocytes and, thus, tissue oxygenation (Siren et al., 2000).

At the same time, the hypoxic damage of neurons is also protected by EPO and it is important to note that EPO production, via HIF-1, is induced not only by hypoxia but also by hypoglycemia and oxidative stress (Chandel et al., 1998 and Fandrey et al., 1997). As demonstrated, these endogenous protective mechanisms are clearly insufficient upon acute injury by the significant effect of exogenously administered EPO in vivo, and it is possible that the brain does not produce enough EPO after cerebral ischemia, or that the latency of neosynthesis is too long to sufficiently protect neural tissue. The inflammatory component of cerebral ischemia may, in fact, limit EPO production; as tumor necrosis factor, whose production in ischemic brain has been
demonstrated (Meistrell et al., 1997), to directly inhibit EPO production in vivo (Davis et al., 1997).

EPO primarily acts to rescue erythroid cells from apoptosis (programmed cell death) and to increase their survival and it also acts synergistically with several growth factors (SCF, GM-CSF, IL-3, and IGF-1) to cause maturation and proliferation of erythroid progenitor cells (primarily colony-forming unit-E). Other effects of EPO include (i) a hematocrit-independent, vasoconstriction-dependent hypertension, (ii) increased endothelin production (iii) up-regulation of tissue rennin (iv) change in vascular tissue prostaglandins production (v) stimulation of angiogenesis and (vi) stimulation of endothelial and vascular smooth muscle cell proliferation (Fisher, 2003).

Necrosis and apoptosis have been proposed as mechanisms of cellular demise and either could be the target of actions of EPO. Following hypoxia, EPO promotes neuronal survival and other metabolic insults by largely unknown mechanisms and the action of EPO is not limited to directly promoting cell survival as EPO is trophic but not mitogenic in cultured neuronal cells (Siren et al., 2000).

History

The existence of erythropoietin (EPO) was first suggested by Paul Carnot and his assistant Deflandre in 1906 and they proposed the idea that hormones regulate the production of red blood cells. They attributed an increase in red blood cells in rabbit subjects to a hemotopic factor called hemopoietin after conducting experiments on rabbits subject to bloodletting. Paul Carnot and Deflandre described the existence of a hormone responsible for erythropoiesis. They have observed that regeneration of blood after bloodletting is under the influence of a humoral process (a process controlled by a substance in the blood) and termed this substance the generic name “Hemopoietine” (http://www.hematology.org/Publications/50-Years-in-Hematology/4740.aspx).

EPO was previously described as a feedback regulator: when the number of red cells was below the normal setpoint, the mechanism to accelerate red cell formation was activated by secretion of EPO. When the set point was regained, the mechanism was thought to turn off, keeping the number of red cells relatively constant. It is now known that normal red cell formation is regulated by continuous secretion of EPO so that the
formation of new red cells matches the loss of old cells. The system, however, can respond to a need for red cells, for example, due to blood loss or hypoxia, by increased secretion of EPO and the consequent increased formation of red cells. (http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B782W-4B0D1X2-2W&_user=10&_origUdi=B6VM6-4G4X02N-CN&_fmt=high&_coverDate=11%2F12%2F2003&_rdoc=1&_orig=article&_origin=article&_zone=related_ref&_acct=C000050221&_version=1&_urlVersion=0&_userid=10&md5=a57e31b5320b710ee3c8j0507b06).

Eva Bonsdorff and Eeva Jalavisto (1948) continued to study the production of red cells and later referred the hemopoietic substance as ‘Erythropoietin’ (EPO) and the existence of EPO was further investigated by Reissman and Erslev (1950). They demonstrated that a certain substance which is circulating in the blood is able to stimulate the red blood cell (RBC) production and increase hematocrit. Further, this substance was finally purified and confirmed as Erythropoietin and that opened the doors to therapeutic uses of EPO in diseases like anemia (Jelkmann, 2007).

The role of the natural hormone EPO in the formation of red blood cells and the various forms of renal failure was studied in 1970, by two scientists, John Adamson, hematologist, and Joseph, nephrologist. In the same decade (1970's) further studies by these two investigators in sheep and other animals, showed that EPO stimulates the production of red cells in bone marrow and could lead to a treatment for anemia in humans. Purification of human EPO was first attempted by Goldwasser and Kung in 1968. They managed to purify in milligrams by 1977 and the pure EPO allowed the amino acid sequence to be partially identified and the gene to be isolated (Jelkmann, 2007). Later, a NIH-funded research program at Columbia University led to the synthesis of EPO. The technique was patented by Columbia University and licensed it to Amgen, an US based company (http://www.titinitorancea.com/erythropoietin.htm).

The contemporary era of EPO research began with the purification of human urinary EPO and, 8 years later, the cloning of its gene. These advancements set the stage for a rapid increase in research and publications on EPO: in the period from 1906 to 1976, there was an average of 16 papers on the subject published per year; from 1976 to 2000, this number increased to 237 per year. It is pertinent to observe that the greater part of this increase was due to the advent of recombinant DNA technology, making it
possible not only to clone the gene and study its expression, but to produce EPO in cells engineered to carry the transgene, harvest EPO, and purify it in industrial amounts for clinical applications. Much of the increase in the number of publications was associated with molecular biology studies and clinical application (http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B782W-4B0DIX2-2W&_user=10&_origUdi=B6VM6-4G4X02NCN&_fmt=high&_coverDate=11%2F12%2F2003&_rdoc=1&_orig=article&_origin=article&_zone=related_ref&_acct=C000050221&_version=1&_urlVersion=0&juserid=10&md5=51c6a57e31b5320b710ee3c8f0507b06).

Human EPO was first isolated and later purified from the urine of aplastic anemia patients by Miyake and Goldwasser in the 1970s. Since then, the structural information and characteristics from the purified material accelerated the cloning of the EPO gene. Several groups devised recombinant DNA methods to produce EPO by the mid-1980s (Lin et al., 1985; Tabbara, 1993). Mammalian cells are essential to produce EPO, because EPO contains 40% carbohydrate that plays some important roles in its activity, stability and biosynthesis. In 1984, two groups succeeded in cloning the EPO gene and expressing this gene in mammalian cells (Inoue et al., 1995).

The first successful clinical trial was conducted for a synthetic form of the hormone, Epogen produced by Amgen, at the Northwest Kidney Centers by Adamson, Joseph, Joan, Michael and Jeffrey in 1980s and the results were published in January 1987 in the New England Journal of Medicine (Eschbach et al., 1987).

The gene encoding human erythropoietin was first isolated by Lin et al., in 1985 from a genomic phage library. They were able to characterize it for research and production of human erythropoietin. Further research demonstrated that the erythropoietin gene encoded the production of EPO in mammalian cells that was biologically active in vitro and in vivo (Vogt et al., 1989). Finally, this had opened up the door for the industrial production of recombinant human erythropoietin (rhEPO) as a therapeutic protein for treating anemia (Lin et al., 1985).

**Structure of EPO**

EPO is a glycoprotein of the class I cytokines family that fold into a compact globular form consisting of four α-helical bundles. The molecular mass of EPO is 30.4
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EPO receptor-binding and signaling events

EPO is a glycoprotein hormone, approximately 34 kDa in size. It consists of a 165 amino acid peptide core that is responsible for receptor-binding and in vitro stimulation of erythropoiesis. The carbohydrate portion, comprising about 40% of the total molecule, is crucial for in vivo survival of EPO. EPO possesses three tetra-antennary nitrogen (N)-linked (at Asn 24, 38 and 83) and one oxygen (O)-linked (at Ser 126) on acidic carbohydrate side chains. While human urinary EPO and the Epoetins are identical with respect to their primary and secondary structure, they exhibit minor differences in their composition of the N- and O-glycans (Jelkmann, 1986).

EPO also exists as a mixture of isoforms like other glycoprotein hormones. This micro-heterogeneity is due to several reasons: i) charged carbohydrate moiety of the protein ii) presence or absence of terminal N-acetyl neuraminic acid residues iii) varying amounts of acetylation iv) presence or absence of N-acetyl lactosamine extensions. Therefore, the electrophoretic mobility and iso-electric point of the molecule is strongly influenced by the degree of sialylation of polysaccharide chains (Lai et al., 1986 and Jelkmann, 1992).

The functional consequences of the micro-heterogeneity of the glycans of endogenous EPO are not well understood. However, the survival of circulating EPO relies on the presence of terminal sialic acid residues of the N-glycans. Since galactose is the pre-terminal sugar of the glycans, de-sialylated EPO is rapidly cleared in the liver via galactosyl receptors of the hepatocytes (Jelkmann, 1986).

EPO receptor binding and signaling events

EPO-responsive cells possess specific transmembrane receptors (EPO-R) that are mainly expressed by the burst-forming units-erythroid (BFU-E) and the colony-forming units-erythroid (CFU-E) in the bone marrow. EPO-R is a homo-dimer of two glycoprotein chains of 484 amino acids. The two EPO-R sub-units usually share one EPO molecule, whereby the dissociation constants for the two binding sites differ greatly. EPO binding induces a conformational change and a tighter association of the EPO-R dimers, as a result, two Janus kinase 2 (JAK2) tyrosine kinases are activated that are in contact with the cytoplasmic region of the EPO-R molecules (Figure 1) (Jelkmann, 1986).
Figure 1: Effects of the Binding of EPO to Its EPO-R, EPO signalling involves autophosphorylation of Janus kinase 2 (JAK2), phosphorylation of EPO receptor (EPO-R), homodimerization of signal transducer and activator of transcription 5 (STAT5), activation of phosphatidylinositol-3-kinase (PI-3K), phosphorylation of the adapter protein Src-homology and collagen (SHC) to form a complex with growth factor receptor binding protein (GRB), son of sevenless (SOS) and the G-protein Ras, and the sequential activation of the serine-kinase Raf, mitogen-activated protein kinase kinase (MEK or MAPKK) and mitogen-activated protein kinase (MAPK). The signaling cascade results in survival, proliferation and differentiation of erythrocytic progenitors. Following de-phosphorylation, the EPO/EPO-R complex is internalized and degraded (Jelkmann W, 1986).
EPO binds to an erythroid progenitor cell surface receptor that includes a p66 chain, and when activated, the p66 protein becomes dimerized. EPO receptor activation induces a JAK2 tyrosine kinase, which leads to tyrosine phosphorylation of the EPO receptor and several proteins. EPO receptor binding leads to intracellular activation of the ras/mitogen-activated kinase pathway, which is involved in cell proliferation, phosphatidylinositol 3-kinase, and STATS 1, 3, 5A, and 5B transcriptional factors (Fisher, 2003).

Few cytokines, such as the interleukins, can affect a relatively wide range of cells and have redundant biological activity as they create redundancy through the common use of a single receptor subunit. Whereas, on the other hand, G-CSF and EPO act with high specificity on a relatively limited range of cells. Therefore, it was probably unnecessary for their receptors to share one of the subunits. EPO on binding to its receptor results in homo-dimerisation of the receptor leading to the activation of several signal transduction pathways- JAK 2/STAT5 system, G-protein (RAS), calcium channel and kinases (Tilbrook and Klinken, 1993; Wen et al., 1994). The effects of EPO on thrombopoiesis may be directly mediated through specific, high-affinity binding sites for EPO on the surface of maturing megakaryocytes (Fraser et al., 1989).

EPO production significantly increases at low oxygen levels and this is the reason that athletes undergo training at high altitudes more specifically to boost EPO production because of the lower oxygen partial pressure. This brings about the desired effect of sustained athletic capability due to a resultant increase in red blood cells (Wide et al., 1989). Similar effect had also occurred naturally in the athlete due to accelerated receptor capability (Yoshimura and Arai, 1996). The receptors do not undergo tyrosine phosphorylation in case of mutations but the activation of JAK2 continues for a longer period of time, and thus the signal is generated more efficiently (Gene et al, 2004). In a mild case of familial erythrocytosis, a mutation was discovered in one patient, where, the C-terminus of the EPO receptor had 70 amino acids missing and the patient’s erythroblasts showed an increased sensitivity to EPO (Chapelle et al., 1993). This was a dominant genetic trait and in this family the impairment was not severe enough to be called an illness. In fact, it is said that this patient was proficient enough athletically to compete for a gold medal at the Olympics.
The phosphorylated tyrosine residues of EPO-R act as docking sites for several signalling proteins. In turn, various kinases, anti-apoptotic proteins and transcription factors are activated. The action of EPO is terminated by the haematopoietic cell phosphatase (HCP or SHP1). On dephosphorylation, the EPO/EPO-R complex is internalised and degraded. It is assumed that EPO-R mediated uptake of by erythropoietic tissues is the major mechanism of the removal of EPO from circulation. In vitro studies have shown that internalization of the EPO/EPO-R complex by the target cells is rapidly followed by its proteasomal and lysosomal proteolysis. Furthermore, the proteasome is responsible for the down-regulation of EPO-R in EPO-stimulated cells, as it prevents the appearance of newly synthesised EPO-R (Jelkmann, 1986).

EPO-R belongs to the cytokine receptor type I super family for which substantial information concerning signaling biology exists (Yoshimura and Misawa, 1998; Wojchowski et al., 1999). Homodimerization on EPO binding activates the receptors in hematopoietic cells, which allows autophosphorylation of EPO-R associated Janus-tyrosine kinase-2 (JAK-2) and JAK-2 activation leads to phosphorylation of several downstream signaling pathways, including ras-mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase [PI(3)K], and the transcription factor STAT5 (Ihle, 1995). The net effect of EPO-R stimulation for erythroblasts is the inhibition of apoptosis, proliferation, and differentiation (Siren et al., 2000).

**EPO in human disease**

The understanding of expression of EPO and EPO-R has a fundamental implication, both in health and disease (http://www.rofar.org/rainbow/portal/alias_rofar/lang_enUS/tabID_3353/DesktopDe fault.aspx). Anemia has a strong incidence both on the quality of life and the evolution of cancer and it may result in cancer from either a bone marrow infiltration of cancer cells or a cytotoxic effect of chemotherapy and/or radiotherapy or both (Milano and Schneider, 2007).

Anemia is a popularly known consequence of chronic kidney disease (CKD) that is characterized by a lack of red blood cells and it has been shown that CKD-related anaemia is due to a decreased production of EPO, a glycoprotein that is the primary regulator of red blood cell production, and is indispensable for terminal differentiation of erythroid progenitors. However, the reasons are poorly understood about this inability to
produce sufficient quantities of EPO. EPO is mostly produced by the liver during embryonic development, but the kidney becomes the main source of EPO after birth and in the liver, EPO is produced by hepatocytes located around the central veins and by stellate cells, while in the kidney it is produced by interstitial cells that have characteristics of fibroblastic cells.

Erythropoietin was first characterized as a hematopoietic growth factor (Jelkmann, 1994) and has been in clinical use by millions of patients over the last decade for the treatment of anemia. EPO and its receptor are observed to be expressed in rodent and human brain tissue (Juul et al., 1998; Marti et al., 1996 and Siren et al., 2001), as well as by cultured neurons (Konishi et al., 1993; Bernaudin et al., 1999; Bernaudin et al., 2000 and Lewczuk et al., 2000) and astrocytes, and that EPO has effects on neuronal cells (Konishi et al., 1993), expanded the biological role of EPO beyond hematopoiesis.

EPO gene expression in the brain is regulated by hypoxia-inducible factor-1 that is activated by a variety of stressors, including hypoxia. It was reported by several independent research groups that EPO protects cultured neurons against glutamate toxicity and reduces ischemic neuronal damage and neurological dysfunction in rodent models of stroke. EPO has neuroprotective role when administered systemically not only in animal models of cerebral ischemia, but also for mechanical trauma, excitotoxins, and neuroinflammation. Marked changes in EPO and EPO receptor (EPO-R) gene expression have been reported to occur in brain tissue after ischemic injury. Biological relevance and specificity of these changes have been demonstrated by the observation that neutralization of endogenous EPO with soluble EPO-R augments ischemic brain damage and thus, it seems that EPO plays a critical role in neuronal survival after hypoxic injury. However, the nature and mechanism of this protective role are currently not clear (Siren et al., 2000).

The common complication of chronic kidney disease is anemia and though mechanisms involved in the pathogenesis of renal anemia include chronic inflammation, iron deficiency, and shortened half-life of erythrocytes, the primary cause is deficiency of erythropoietin (EPO). The EPO levels in serum of patients with chronic kidney disease are usually within the normal range and thus fail to show an appropriate increase.
with decreasing hemoglobin levels, as found in non-renal anemias (Masaomi and Kai-Uwe, 2006).

EPO is presently considered to have applicability in a variety of nervous system disorders that can overlap with vascular disease, metabolic impairments, and immune system function and is no longer believed to have exclusive biological activity in the hematopoietic system. As a result of this, EPO may offer efficacy for a broad number of disorders that involve Alzheimer's disease, cardiac insufficiency, stroke, trauma, and diabetic complications and during a number of clinical conditions. EPO is robust and can prevent metabolic compromise, neuronal and vascular degeneration, and inflammatory cell activation. Yet, use of EPO is not without its considerations especially in light of frequent concerns that may compromise clinical care (Kenneth et al., 2008).

Studies elucidating the regulation of EPO expression led to the identification of the hypoxia inducible factor–hypoxia responsive element system. However, the reason why EPO production is inappropriately low in diseased kidneys remains incompletely understood despite much progress in understanding the molecular mechanisms through which cells can sense oxygen availability and translate this information into altered gene expression. Alterations in the function of EPO-producing cells as well as perturbations of the oxygen-sensing mechanism in the kidney may contribute. As with other anemias, the consequences of renal anemia are a moderate decrease in tissue oxygen tensions and counter regulatory mechanisms that maintain total oxygen consumption, including a persistent increase in cardiac output (Masaomi and Kai-Uwe, 2006).

A number of novel pathways (cellular) governed by EPO that can open new avenues to avert deleterious effects of this agent and offer previously unrecognized perspectives for therapeutic strategies have been elucidated based on the work reported in the recent past. Elucidating its unique cellular pathways and obtaining greater insight into the role of EPO in the nervous system may provide greater cellular viability not only in the nervous system but also throughout the body (Kenneth et al., 2008).
Recombinant human erythropoietin as therapeutic protein

Recombinant human erythropoietins (rhEPO) as well as other erythropoiesis stimulating agents (ESAs) are now widely used to treat anemia associated with a range of conditions, including chronic kidney disease, chronic inflammatory disorders and cancer. There is also increased awareness of the potential abuse of ESAs to boost athletic performance in competitive sport and the discovery of erythropoietin receptors outside of the erythropoietic compartment may herald future applications for ESAs in the management of neurological and cardiac diseases. The present controversy concerning optimal hemoglobin levels in chronic kidney disease patients treated with ESAs and the potential negative clinical outcomes of ESAs treatment in cancer reinforces the need for cautious evaluation of the pleiotropic effects of ESAs in non-erythroid tissues (Vivien et al., 2007).

Recombinant human EPO (rhEPO) is currently being used to treat patients with anemias associated with chronic renal failure, AIDS patients with anemia due to treatment with zidovudine, non-myeloid malignancies in patients treated with chemotherapeutic agents, perioperative surgical patients, and autologous blood donation (Fisher, 2003). EPO has revolutionized the treatment of anemia of chronic renal failure and it has been shown to decrease hospitalizations, increase survival, improve brain and cognitive function and finally improved the quality of life of renal patients (Tong and Nissenson, 2001).

The commercial EPO agents (also known as erythropoietic agents) already in use include Epoetin alfa, Epoetin beta, and Darbepoetin alfa. Proline hydroxylase inhibitors or CERA (Continuous Erythropoietin Receptor Activator) or that increase Hypoxia Inducible Factor-1 (HIF-1) thereby stimulating EPO production and iron availability and supply are the newer agents that are under active investigation. ESAs have been found to promote neuronal regeneration and to decrease post-stroke infarct size in mouse models and they have also been reported to shorten survival when used to treat anemia in many cancer patients and to increase thromboembolism (Neeraj and Josef, 2008).

Replacement therapy with recombinant human EPO (rhEPO) efficiently restores the blood haemoglobin concentration in most pre-dialysis and dialysis renal failure
patients (Jelkmann, 1986). Based on various animal studies, it was concluded that recombinant EPO, which is available as a pharmaceutical agent, exerts significant cardio-protective effects against infarction and ischemia reperfusion injury, also against non-ischemic cardiac dysfunction (http://www.rofar.org/rainbow/portal/alias_rofar/lang_en-US/tabID_3353/DesktopDefault.aspx).

**Industrial production of rhEPO**

EPO quickly made its way into the market as a therapeutic protein due to its therapeutic demand, worldwide, for the treatment of anemia resulting from a host of conditions, primarily kidney failure, HIV infection in patients treated with AZT, and anti-cancer chemotherapeutic drugs (Schellekens and Ryff, 2002). Therefore, many pharmaceutical industries had shown interest in producing recombinant human erythropoietin and soon it was brought into the market by many national and international companies (Table 1) (http://www.steroidreport.com/2008/07/21/biosimilar-epo-agents).

Recombinant human EPO is most commonly obtained from transfected clones of Chinese hamster ovary (CHO) cells but also from other sources, such as recombinant baby hamster kidney (BHK) cells (Hammerling et al., 1996).
Table 1: Some of the major rhEPO producing National and International companies

(http://www.steroidreport.com/2008/07/21/biosimilar-epo-agents)

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Company</th>
</tr>
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<tbody>
<tr>
<td><strong>Indian</strong></td>
<td></td>
</tr>
<tr>
<td>Zyrop</td>
<td>Zydus Biogen, Ahmedabad</td>
</tr>
<tr>
<td>Epofit</td>
<td>Intas Biopharmaceuticals Ltd., Ahmedabad</td>
</tr>
<tr>
<td>Erykine</td>
<td>Intas Biopharmaceuticals Ltd., Ahmedabad</td>
</tr>
<tr>
<td>Hemax</td>
<td>Hindustan antibiotics, Pune</td>
</tr>
<tr>
<td>Shanpoeitin</td>
<td>Shanta Biotechnics, Hyderabad</td>
</tr>
<tr>
<td>Wepox</td>
<td>Wockhardt, Mumbai</td>
</tr>
<tr>
<td><strong>International</strong></td>
<td></td>
</tr>
<tr>
<td>Eprex</td>
<td>Cilag, Schaffhausen, Switzerland</td>
</tr>
<tr>
<td>Neo recomon</td>
<td>Roche, Basel, Switzerland</td>
</tr>
<tr>
<td>Epogen</td>
<td>Amgen, California, USA</td>
</tr>
<tr>
<td>Procit</td>
<td>Amgen, California, USA</td>
</tr>
<tr>
<td>Ceriton</td>
<td>Ranbaxy, New Jersy, USA</td>
</tr>
</tbody>
</table>
Erythropoietin which is produced in mammalian cell lines by recombinant DNA technology is available in the market as a therapeutic agent and is used in treating anemia resulting from myelodysplasia and chronic kidney disease from the treatment of cancer (chemotherapy and radiation), and from other critical illnesses (heart failure). It is available in several forms in the market as biomedicine namely, Epogen, Darbepoetin Epotin, Betapoietin, Eprex, Relipoietin, Epokine, Methoxy Polyethylene Glycol-Epoetin Beta, etc (http://www.steroidreport.com/2008/07/21/biosimilar-epo-agents). The main applications of rhEPO as a therapeutic agent are as follows:

- Treatment of acute ischemic stroke (http://books.google.com.au/books?id=A76u7g0QnskC)
- Anemia due to chronic kidney disease and Lung disease (Macdougall et al., 1996 and Miller et al., 1981)
- Neuroprotection (Ehrenreich et al., 2004 and Juul, 2004)
- Anemia due to treatment for cancer (Schrijvers et al., 2010 and Buemi et al., 2005)
- Retinal survival factor (Becerra and Amaral, 2002)
- Anemia in critically ill patients (Corwin et al., 2007)
- Wound healing (Haroon et al., 2003)

Recombinant human EPO synthesized by recombinant CHO cells is secreted into the culture medium (supernatant) from where it is purified by means of various chromatographic methods until the desired purity is achieved. In the industrial production conditions of EPO, the recombinant CHO cells are grown in roller bottles and the resulted rhEPO has a similar biological and chemical characteristics like that of international reference standard available for EPO. The isolation of human EPO encoding cDNA clones and their subsequent expression in eukaryotic cells has enabled the production of unlimited amounts of this hormone (Lee, 1984).

The human EPO gene is on chromosome 7 (7pter–q22) and is a 2144 bp stretch of DNA. The structural gene consists of four introns, comprising 1562 bp, and five exons, totaling 582 bp. There is an 81 bp sequence coding for a signal peptide immediately downstream of the ATG start site. Upstream of the signal sequence there do not appear to be any canonical promoter sequences. Downstream of the termination site, there is an important sequence defining the enhancer, hypoxia-response element, coding
for hypoxia-inducible factor-1. There is little if any homology between the EPO gene and the sequences of other mammalian genes except for these regulatory elements in the 30'-flanking region (http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B782W-4B0D1X2-2W&_user=10&_origUdi=B6VM6-4G4X02N-CN&_fmt=high&_coverDate=11%2F12%2F2003&_rdoc=1&_orig=article&_origin=article&_zone=related_ref&_acct=C000050221&_version=1&_urlVersion=0&_userid=10&md5=51c6a57e31b5320b710ee3c8f0507b06).

The gene of rhEPO was cloned and inserted into an eukaryotic expression vector. CHO (Chinese hamster ovary) cells were transfected with this vector carrying the human EPO gene and further selection was performed to select a clone for its high expression level and growth capacity. The N-glycosylated moiety of recombinant human erythropoietin has three main functional units; the main core, the branched portion and the terminal component with each unit having a specific role. The function of the O-glycosylated unit, a component constituting about 3% of the total mass of recombinant human erythropoietin, remains to be defined (Ng et al., 2003).

The amino acid sequence of human EPO shows it to consist of approximately 34% hydrophobic amino acids. There is little significant homology between the amino acid sequence of human EPO and that of any other human hormone, cytokine, or growth factor (http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B782W-B0D1X2-2w&_user=10&_origUdi=B6VM6-4G4X02NCN&_fmt=high&_coverDate=11%2F12%2F2003&_rdoc=1&_orig=article&_origin=article&_zone=related_ref&_acct=C000050221&_version=1&_urlVersion=0&_userid=10&md5=51c6a57e31b5320b710ee3c8f0507b06).

The common strategy used for the production of a recombinant human erythropoietin using a recombinant CHO cell line includes the following:

- Human EPO gene isolation and cloning
- Isolation of rhEPO-producing CHO cells
- Control of the EPO mRNA produced by the rhEPO-CHO cells
- Sequencing of the EPO cDNA cloned in a vector
**Human EPO gene isolation and cloning**

The human EPO gene was obtained from genomic human DNA by PCR (Polymerase Chain Reaction) amplification. It was cloned in a plasmid vector for its sequencing, sub-cloned in a eukaryotic expression vector, co-transfected with another plasmid containing the DHFR gene, in Chinese Hamster Ovary cells (CHO). Genetic amplification was made using MTX and the EPO producing cell clones were selected by ELISA test. Total RNA was isolated from EPO producing cells and amplified by PCR. The amplified product was cloned and sequenced to compare the isolated gene with that of the EPO gene sequence documented in Gene Bank (http://www.ncbi.nlm.nih.gov/nuccore/182197?report=fasta Gene Bank: Accession No.M11319).

CHO cell line mutated to be deficient in the DHFR gene (CHO- DHFR) in order to facilitate genetic amplification with MTX. The cells were co-transfected, by the calcium phosphate technique (Jordan et al., 1996).

**Isolation of rhEPO- producing CHO cells**

Clones that grew in presence of MTX were isolated, amplified in fresh medium and once grown; the culture supernatant was tested to measure the rhEPO secretion using a commercial ELISA test kit (R&D Systems Inc., MN, USA).

**Control of the EPO mRNA produced by the rhEPO-CHO cells**

- Preparation of cell RNA
- Preparation of specific DNA:
- Amplification and cloning of EPO cDNA

**Sequencing of the EPO cDNA cloned in pUC18 vector**

The emergence of the biotechnology industry two decades ago sparked the development of methods for large-scale cell culture, as companies raced to produce therapeutic quantities of the first recombinant proteins. Continuous refinements to cell culture techniques, instrumentation, and quality control measures ultimately made large-scale cell culture more a science than an art (Glazer, 2001).

Mammalian cells have grown reliably, robustly, and productively in culture for years in tried and-true serum-supplemented media. Expiry of rhEPO patent (www.open.imshealth.com), demand for rhEPO in the worldwide market (Figure 2) and its projected sales in the present and future market (Figure 3) had enabled the production of rhEPO by many companies (Table 1). Since, the technology of manufacturing employed in almost all the companies are similar, in order to cater the market needs, cost reduction is the only alternative, which can be accomplished by eliminating the use of serum during growth phase of the cells.

Chinese Hamster Ovary (CHO) cells are of great interest for bio-processing and pharmaceutical research and development. It’s a well known fact that these cells are able to produce a variety of recombinant glycoproteins at high levels on a large scale due to their robust nature in culture. However, different cell clones of CHO often possess diverse nutritional requirements that are unique to each clone. As a result, optimization of medium for CHO cells can be very challenging, often requiring the development of a custom medium for each particular clone (http://www.sigmaaldrich.com/life-science/cell-culture/serum-free-media/protein-expression-cho.html).

The ideal media should support fast growth. But once the confluency is achieved, cells should stop dividing and produce the product. It is typically observed that about 25% of media support good growth and productivity, while 25 % resulted in no growth at all and about 50% lie in between these two extremes. Cost of manufacturing is high by mammalian cell culture and is the main reason for the high cost of many biologics in the market. At present, there are many approved bio-therapeutics in the market some of which can be life saving too. But high cost may be keeping it from the reach of many patients. The challenge to the industry is to bring the cost down to be affordable to all needy patients and a significant saving in terms of cost can be achieved by following a small improvement in productivity of a cell line (Angell, 2005).
Figure 2: EPO market distribution (%) worldwide
(www.open.imshealth.com)
Figure 3: EPO projected sales from the year 2004 to 2010
(www.open.imshealth.com)
In addition to cost reduction, problems such as lot-to-lot inconsistency, introduction of adventitious agents and presence of proteins, limit widespread use of foetal bovine serum (FBS) in cell culture system. Viral, bacterial and fungal contamination of serum has for some years been a concern by manufacturers of biopharmaceuticals. This has been a driving force behind the adoption of serum-free formulations in the manufacturing process (http://www.athenaes.com/tech_brief_serum_free.php). Therefore, many studies have been performed during the recent past aiming to reduce or eliminate serum requirements. (Batista et al., 2006). These have included the use of poorly defined supplements (for example, pituitary extracts, chick embryo extracts, bovine milk fractions, bovine colostrums), and various plant extracts (for example, vegetal serum). In some cases, it is possible to use fully chemically defined media with appropriate hormones and growth factors (Sandra et al., 2005).

Addition of serum delivers many advantages to cell culture but it also brings with it many important draw backs such as safety issues and batch-to-batch variability during manufacturing. Therefore, researchers believed that use of serum-free medium reduces operating costs and process variability and removes a potential source of infectious agents. Adventitious agents such as bovine spongiform encephalopathies and foot and mouth disease are a major safety concern during manufacturing of biologics. This has led researchers to remove serum and products of animal origin from cell culture media during manufacture of protein based biologics (http://www.biospectrumasia.com/content/130808ind6802.asp).

Anchorage (attachment) dependent cell lines require an extracellular matrix on the growth substratum. As serum provides some of the components for this matrix, when using serum-free medium the substratum (plastic dishes) should be pre-coated with a fibronectin, laminin or another suitable alternative (http://www.dnr-is.com/src/SFM%20General(13).pdf).

Use of hormonally defined media for culture of cells has allowed (a) the demonstration of physiological responses from cells usually unable to express them in vitro and (b) the study of the effects on growth and differentiation of diffusible factors and attachment factors. The embryonal carcinoma line 1003, when grown in serum-containing medium, forms multidifferentiated tumors in vivo but was unable to
differentiate in vitro and in a defined medium containing insulin, transferrin, selenium, and fibronectin as attachment factors, 1003 cells grow for several generations and differentiate into neurons and embryonic mesenchyme (Michel, 2007).

The classical media (basal) used in manufacturing such as RPMI, IMDM or DMEM are supplemented with 5 % to 20 % serum. Initially, because of the cost and process constraints of serum, serum free medium was introduced into biopharmaceutical manufacturing and Ham first used serum free medium in mammalian cell culture in 1965 (http://www.antibodyresearch.com/index.php?option=com_myblog&show=serum-free-media-all-are-not-created-equal.html&Itemid=41). Henceforth, many alternatives have been screened to substitute serum.

Milk is one of the most nutrient dense foods. The important constituents of milk include suspended and dissolved proteins, fats, carbohydrates, vitamins and minerals in addition to water, which constitutes the vital elements required for growth. A number of milk types and milk fractions were investigated as possible substitutes for serum in cell culture media. A filtrate of reconstituted non-fat dry milk showed a potential application. Culture fluids containing 5% of the non-fat dry milk filtrate were used to propagate primary and continuous cell cultures. The cell culture growth from these was compared with that of cells grown in a serum-containing medium. The non-fat dry milk filtrate-supplemented medium supported the growth of all epithelial cells tested. The non-fat dry milk filtrate is inexpensive, easy to procure, easily prepared, and is a potential substitute for serum in cell culture (Fassolitis et al., 1981).

Earlier, Baron et al., (1958) developed a serum free maintenance medium containing autoclaved skim milk that effectively maintained a number of cell cultures (Gochenour and Baron, 1959). Rabson and Legallais (1959) used a serum free medium containing autoclaved skim milk, which supported the growth of a cells recovered form a human epidermoid carcinoma. Various milks and milk fractions have been investigated as replacements for serum in cell culture media. Although there was an initial lag period when cells were propagated in a medium containing the raw milk filtrate, cells became confluent in 10 to 14 days, and the average split on passage was 1 to 3. A non-fat dry milk filtrate was found to possess similar properties and requires no de-fatting or centrifugation (Rabson and Legallais, 1959).
1.0 Introduction

The use of milk and milk whey, as nutritional components (Bury et al., 1998; Steimer and Klagsbrun, 1981; Steimer et al., 1981; Ramirez et al., 1990 and Batista et al., 2005), for normal, transformed and established cell lines could reduce the consumption of serum required for the growth of cells up to 90% (Saha and Sen, 1989).

There are two approaches to adapt cells to serum-free medium which are as follows,

- **Direct adaptation**, in which cells are switched from serum-supplemented medium into serum-free medium (SFM).
- **Sequential adaptation** or **weaning**, wherein cells are switched from serum supplemented medium into SFM in several steps.

Sequential adaptation tends to be less harsh on cells than direct adaptation (www.invitrogen.com).

This doctoral dissertation envisages the application of serum free growth medium for the production of recombinant human erythropoietin from recombinant chinese hamster ovary cells as an alternative for serum based growth medium.