การดัดแปลงหมู่ไลซีนบนโครงสร้างโมเลกุลของฮอร์โมนอิริโทรพอยอิตินด้วยปฏิกิริยาเคมีและการทดสอบฤทธิ์ในการกระตุ้นการสร้างเม็ดเลือดแดง และฤทธิ์ในการปกป้องเนื้อเยื่อไต

นางสาวสุปรีชา ฉัตรทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CHEMICALLY TARGETED-LYSINE MODIFICATIONS OF ERYTHROPOIETIN: ERYTHROPOIESIS AND RENOPROTECTIVE EFFECT OF LYSINE MODIFIED ERYTHROPOIETIN MOLECULES

Miss Supreecha Chattong

A Dissertation Submitted in Partial Fulfillment of the Requirements

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(Interdisciplinary Program)

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Thesis Title	CHEMICALLY TARGETED-LYSINE MODIFICATIONS OF ERYTHROPOIETIN: ERYTHROPOIESIS AND RENO - PROTECTIVE EFFECT OF LYSINE MODIFIED-ERYTHROPOIETIN MOLECULES
By	Miss Supreecha Chattong
Field of Study	Biomedical Science
Thesis Advisor	Assistant Professor Amornpun Sereemaspun, M.D., Ph. D.
Thesis Co-advisor	Krissanapong Manotham, M.D., Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Graduate School

(Associate Professor Amorn Petsom, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Chintana Chirathaworn, Ph.D.)

.....Thesis Advisor

(Assistant Professor Amornpun Sereemaspun, M.D., Ph.D.)

.....Thesis Co-advisor

(Krissanapong Manotham, M.D., Ph.D.)

.....Examiner

(Professor Somchai Eiam-Ong, M.D., Ph.D.)

.....Examiner

(Assistant Professor Rachaneekorn Tammachote, Ph.D.)

.....External Examiner

(Assistant Professor Wisit Tangkeangsirisin, Ph.D.)

สุปรีชา ฉัตรทอง : การดัดแปลงหมู่ไลซีนบนโครงสร้างโมเลกุลของฮอร์โมนอิริโทรพอย-อิตินด้วยปฏิกิริยาเคมีและการทดสอบฤทธิ์ในการกระตุ้นการสร้างเม็ดเลือดแดง และฤทธิ์ในการปกป้องเนื้อเยื่อไต. (CHEMICALLY TARGETED-LYSINE MODIFICATIONS OF ERYTHROPOIETIN: ERYTHROPOIESIS AND RENOPROTECTIVE EFFECT OF LYSINE MODIFIED ERYTHROPOIETIN MOLECULES)อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.นพ.อมรพันธุ์ เสรีมาศพันธุ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : นพ. กฤษณพงศ์ มโนธรรม, 60 หน้า.

รีคอมบิแนนท์อิริโทรพอยอิตินถูกใช้เป็นยาหลักในการรักษาโรคโลหิตจาง ซึ่งเป็นภาวะแทรกซ้อนที่พบได้บ่อยในผู้ป่วยไตวายเรื้อรัง ในปัจจุบันนี้มีงานวิจัยจำนวนมากแสดงให้เห็นว่ารีคอมบิแนนท์อิริโทรพอยอิตินสามารถออกฤทธิ์ปกป้องเนื้อเยื่อจากพยาธิสภาพต่าง ๆ แต่การใช้รีคอมบิแนนท์อิริโทรพอยอิตินเพื่อปกป้องเนื้อเยื่อในผู้ที่ไม่มีภาวะโลหิตจาง จะทำให้เกิดการเพิ่มของเม็ดเลือดแดงมากเกินปกติจนเป็นอันตรายได้ คาร์บามัยเลทเตดอิริโทรพอยอิตินเป็นอนุพันธ์ชนิดแรกของอิริโทรพอยอิตินที่มีฤทธิ์ในการปกป้องเนื้อเยื่อจากการบาดเจ็บ แต่สูญเสียฤทธิ์ในการกระตุ้นการสร้างเม็ดเลือดแดง ในการทดลองนี้ได้การดัดแปลงหมู่ไลซีนบนโครงสร้างโมเลกุลของฮอร์โมนอิริโทรพอยอิตินด้วยปฏิกิริยาเคมี เพื่อสร้างอนุพันธ์ตัวใหม่ และทำการทดสอบฤทธิ์ในการกระตุ้นการสร้างเม็ดเลือดแดง และฤทธิ์ในการปกป้องเนื้อเยื่อไต จากการทดลองนี้สามารถสร้างกลูตาราลดีไฮด์อิริโทพอยอิตินเป็นอนุพันธ์ตัวใหม่ เมื่อทดสอบฤทธิ์ในการกระตุ้นการสร้างเม็ดเลือดแดงพบว่า กลูตาราลดีไฮด์อิริโทพอยอิตินไม่มีฤทธิ์ในการกระตุ้นการสร้างเม็ดเลือดแดงในหนูทดลองและเมื่อทดสอบฤทธิ์ในการปกป้องเนื้อเยื่อ พบว่าอนุพันธ์ใหม่นี้มีฤทธิ์ในการปกป้องเซลล์จากกระบวนการตายแบบอพอพโทซิสในเซลล์ไลน์ชนิด HEK-293 และ P19 และ สามารถปกป้องไตในหนูทดลองที่ชักนำให้เกิดภาวะไตขาดเลือดได้ จากการศึกษาลักษณะสมบัติพบว่า โมเลกุลกลูตาราลดีไฮด์อิริโทพอยอิตินมีประจุบวกลดลงเมื่อเทียบกับอิริโทรพอยอิติน การตกตะกอนด้วยแอนติบอดีแสดงให้เห็นว่าโมเลกุลกลูตาราลดีไฮด์อิริโทพอยอิตินจับกับ βCR/EPOR heterotrimeric receptorโดยการออกฤทธิ์ในการปกป้องเนื้อเยื่อจะเกิดผ่านการกระตุ้น Bcl-2

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

5387877120 : MAJOR BIOMEDICAL SCIENCE
KEYWORDS : ERYTHROPOIETIN / NON-ERYTHROPOIETIC / LYSINE MODIFICATION / RENOPROTECTIVE

SUPREECHA CHATTONG : CHEMICALLY TARGETED-LYSINE MODIFICATIONS OF ERYTHROPOIETIN: ERYTHROPOIESIS AND RENO-PROTECTIVE EFFECT OF LYSINE MODIFIEDERYTHROPOIETIN MOLECULES. ADVISOR : ASSIST. PROF. AMORNPUN SEREEMASPUN, M.D. Ph.D., 60 pp.

Recombinant human erythropoietin (rHuEPO) is currently used for renal anemia treatment. Recently, rHuEPO has been shown to provide pleiotrophic tissue protection in various pathologic conditions. The benefits of rHuEPO beyond anemia treatment are limited since it increases red blood cell mass. Carbamylated erythropoietin (CEPO) is the first rHuEPO derivative that lacks erythropoietic activity, but retains tissue protection properties. Since carbamylation targets lysine residues on rHuEPo, we hypothesized that targeted-lysine modifications of rHuEPO may result in a novel non-erythropoietic erythropoietin. rHuEPO was subjected to various targeted-lysine modifications. In vitro cytoprotection and apoptosis were evaluated using P19 and HEK293 cells. In vivo erythropoiesis was performed by administrating these derivatives into animals for two weeks. Renoprotection was tested on ischemia/reperfusion (I/R) model. We here synthesized a novel derivative, a glutaraldehyde erythropoietin (GEPO). This derivative abolishes in vivo erythropoiesis. In vitro experiments showed that GEPO had the cytoprotective activities in both P19 and HEK293 cells In vivo experiments also showed that GEPO ameliorated kidney damage due to I/R injury in both functional and histological aspects. Biochemical characterization showed that GEPO was more electrostatically negative than rHuEPO. Immunoprecipitation revealed that GEPO bound to the βCR/EPOR heterotrimeric receptor and ameliorated cellular apoptosis via the activation of Bcl-2.

Field of Study : Biomedical Science Student's Signature .....

Academic Year : 2012 Advisor's Signature .....

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LIST OF ABBREVIATION

AEPO	=	Acetylated EPO
βCR	=	beta common receptor
CEPO	=	Carbamylated erythropoietin
CFU	=	Colony forming unit
EPO	=	Erythropoietin
EPOR	=	Erythropoietin receptor
GEPO	=	Glutaraldehyde erythropoietin
GuEPO	=	Guanidinated erythropoietin
Ht	=	Hematocrit
HEK-293 cells	=	Human embryonic kidney cells
I/R	=	Ischemia/Reperfusion
ISP	=	Isoelectric points
LDH	=	Lactate dehydrogenase
NSS	=	Normal saline
PAGE	=	Polyacrylamide gel electrophoresis
PEPO	=	Phosphopyridoxylated EPO
PVDF	=	Polyvinylidene fluoride
rHuEPO	=	Recombinant human erythropoietin
SCr	=	Serum creatinine
SEPO	=	Succinylated EPO

TNBS	=	Trinitrobenzenesulfonic

CHAPTER I

INTRODUCTION

Background and Rationale

Erythropoietin (EPO), a 165-amino-acid glycoprotein, regulates red blood cell maturation by promoting the viability, proliferation and differentiation of erythrocyte progenitor cells in bone marrow. In adult, circulating EPO is produced by peritubular fibroblast-like cells in the kidneys and function as hormone[1-4]. It is now clear that EPO is also produced locally by many other tissues as a defensive paracrine signal in response to multiple insults [5-7]. Moreover, administration of recombinant human EPO (rHuEPO) has been reported to protect various organs, including the kidney, from injuries [8-13]. With these evidences, it is clear that EPO is a pleiotropic cytokine for tissue protection, and the use of rHuEPO as an adjunctive treatment in various diseases seems promising [14-15]. However, to elicit tissue protection, one has to administer exogenous rHuEPO at a relatively high dose which, in turn, will be complicated by pathologic increases in red blood cell mass, i.e. polycythemia. This drawback limits clinical use of rHuEPO for tissue protection; therefore a non-erythropoietic EPO that provides tissue protection is strongly required.

Recently, carbamylated EPO (CEPO) has been created by subjecting rHuEPO to cyanide carbamylation (in strict terminology; *carbamoylation*) [16]. This molecule is the first modified rHuEPO to provide tissue protection without significantly increased red blood cell production [17-23]. Further studies suggest that CEPO may not mediate its actions by the conventional homodimerization of EPO receptors (EPOR), but by heterotrimerization between one EPOR and two IL-3 common beta receptors (βCR) [24]. The carbamylation reacts on free amino acids; thus, eight lysine residues and one terminal amino acid on EPO are potential targets. Previous data also hinted that the electric charge on lysine residues of EPO molecule was critical

in mediating the erythropoietic effect [25]. Neutralizing lysine positive charge, for example, by acetylation, carbamylation or making the lysine charge negative through succinylation, caused a significant decrease in erythropioesis. Whether these lysine modified EPOs retain tissue protective activities, remains to be elucidated. In addition, it should be possible to obtain novel non-erythropiotic EPOs with therapeutic potential by targeting lysine modification with other methods as well.

Research questions

1. Do targeted-lysine modifications of rHuEPO result in novel non-erythropoietic erythropoietin ?
2. Do these derivatives exert no erythropoietic properties, but retain cell-protective characteristics both *in vitro* and *in vivo* ?

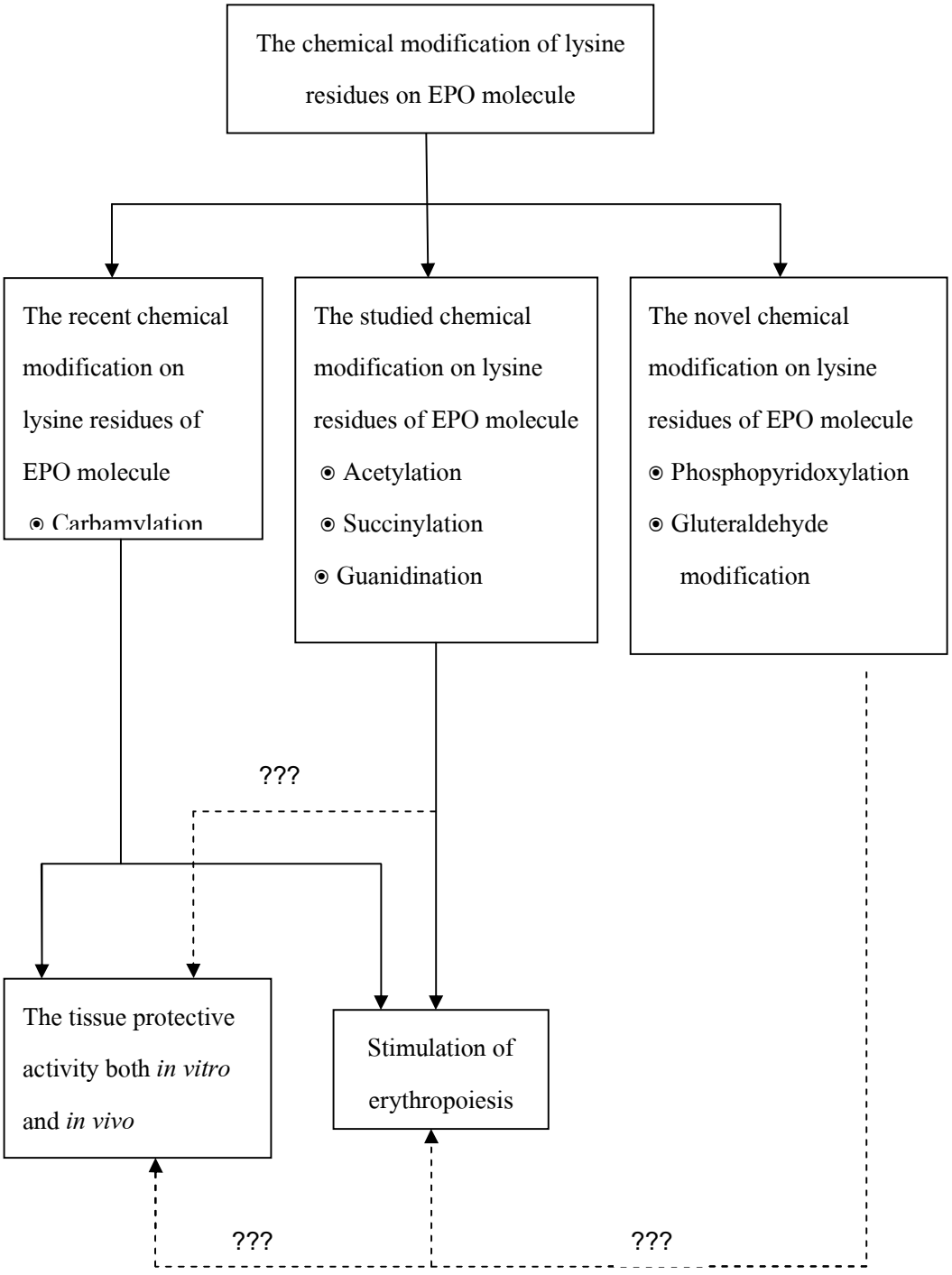
Objectives

1. To synthesize lysine –modified EPOs.
2. To study the cytoprotection and erythropoietic activity of these modified EPOs.
3. To obtain novel non-erythropiotic EPOs with therapeutic potential by targeting lysine modification with other methods.

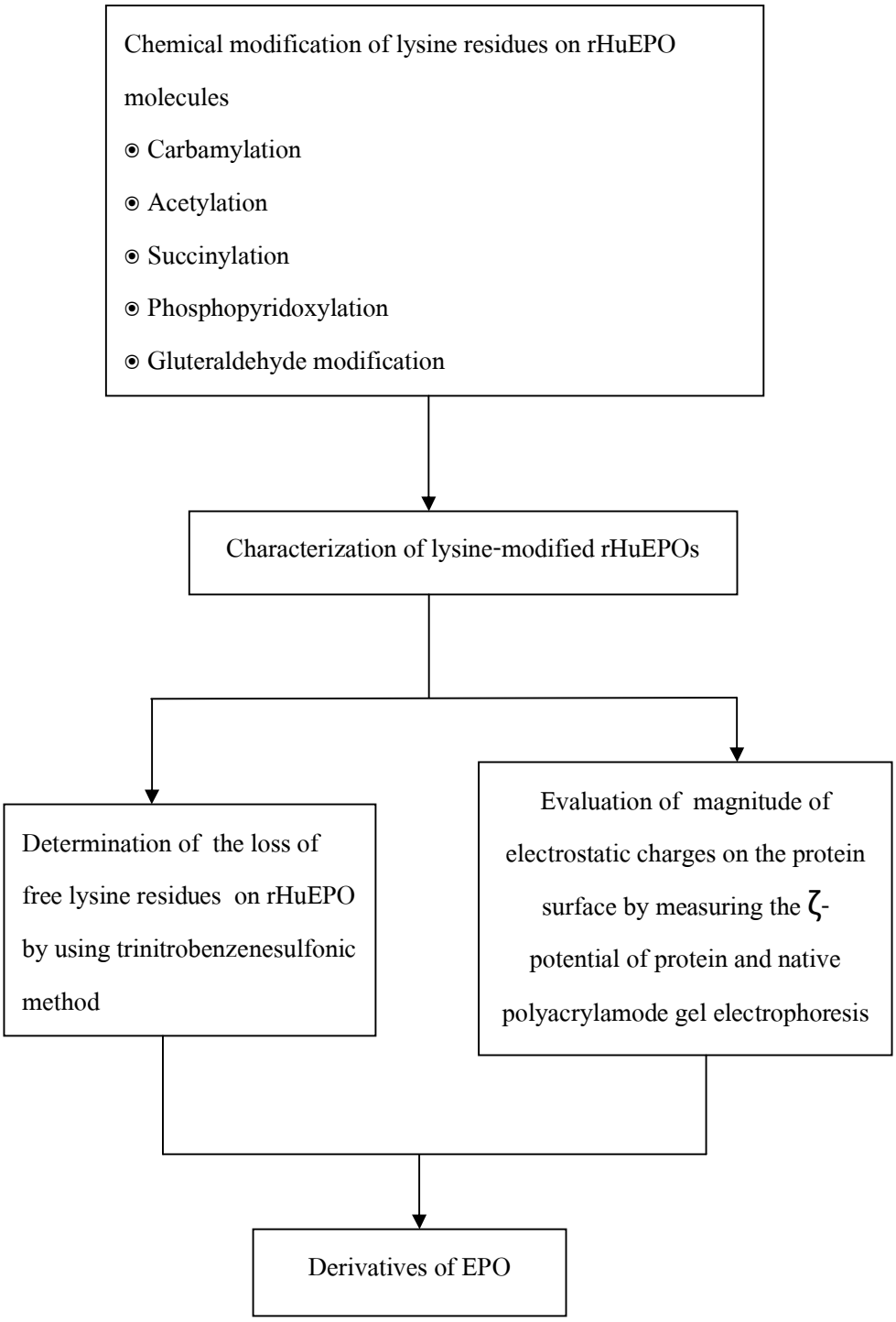
Hypotheses

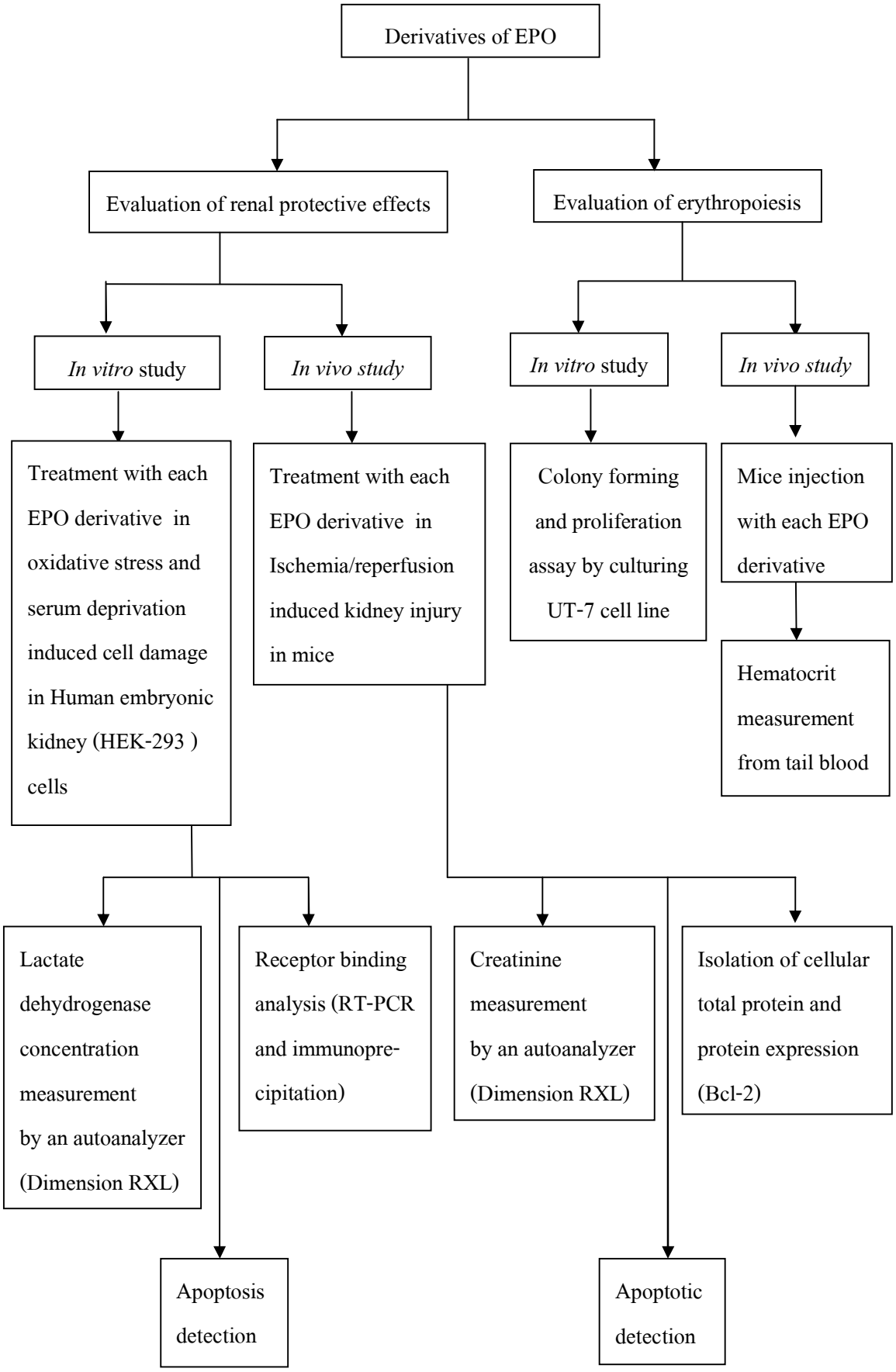
1. Targeted-lysine modifications of EPO molecules cause a loss of erythropoietic effect.
2. The chemical modification on lysine residues of rHuEPO may exert no erythropoietic properties, but retains renal cell-protective characteristics both *in vitro* and *in vivo*.

Conceptual framework



Experimental design




Derivatives of EPO
Evaluation of renal protective effects
Evaluation of erythropoiesis
In vitro study
In vivo study
In vitro study
In vivo study
Treatment with each EPO derivative in oxidative stress and serum deprivation induced cell damage in Human embryonic kidney (HEK-293) cells
Treatment with each EPO derivative in Ischemia/reperfusion induced kidney injury in mice
Colony forming and proliferation assay by culturing UT-7 cell line
Mice injection with each EPO derivative
Hematocrit measurement from tail blood
Lactate dehydrogenase concentration measurement by an autoanalyzer (Dimension RXL)
Receptor binding analysis (RT-PCR and immunopre-cipitation)
Creatinine measurement by an autoanalyzer (Dimension RXL)
Isolation of cellular total protein and protein expression (Bcl-2)
Apoptosis detection
Apoptotic detection

Expected benefits and applications

This study aims to obtain novel non-erythropiotic EPOs that provides tissue protection by targeting lysine modification with other methods. This EPO derivative will eliminate the drawback in clinical use of rHuEPO for tissue protection. The results from this study will benefit for future use as an adjunctive treatment of kidney disease pateint to receive new alternately effective therapeutic intervention.

CHAPTER II

REVIEW OF RELATED LITERATURES

Biology of erythropoietin

The glycoprotein hormone erythropoietin (EPO) is an important survival and growth factor for the erythrocytic progenitors in bone marrow and other haemopoietic tissues. EPO prevents the apoptotic cell death of erythroid colony forming units (CFU-Es), promotes their proliferation and differentiation to yield a herd of pro-normoblasts, and accelerates the development of reticulocytes by shortening the transition time through the pro-normoblast and normoblast stages. As it takes several days for the CFU-Es to produce the progeny of reticulocytes, a significant increase in haematocrit (Hct) is usually not observed earlier than about 2 weeks after a rise in the plasma EPO concentration. Lack of EPO results in anaemia. [1,2,16]

Kidneys are the main sites for the production of EPO in adult humans [1]. Here, and in other EPO producing organs, such as liver [4], the rate of the expression of the EPO gene depends on the level of tissue oxygenation through the availability of the hypoxia-inducible transcription factor 2 (HIF-2). Plasma EPO level can be increased up to three orders of magnitude above the normal value of about 0.015 units/ml (U/ml) in anaemia or hypoxaemia. This response is missing in patients with the anaemia of chronic kidney disease (CKD). The aetiology of the anaemia of inflammation and cancer is more multifactorial, as it involves a relative lack of EPO, insufficient iron availability as a result of the blocking action of hepcidin, inhibition of the proliferation of the erythrocytic progenitors by cytokines, shortened red blood cell (RBC) survival and, possibly, bleeding [25].

Recombinant human erythropoietin has been used for treatment of renal anaemia for almost 20 years [26]. The first-generation rHuEPOs have been engineered in clones of Chinese hamster ovary (CHO) cell lines transfected with human EPO gene, respectively with EPO cDNA

[27]. Following the cloning of the human EPO gene (EPO) and its in vitro expression, recombinant human EPO (rHuEPO) was initially licensed for the treatment of predialysis and dialysis CKD patients and, more recently, cancer patients receiving chemotherapy for solid tumours, malignant lymphomas or multiple myelomas. The therapy with rHuEPO and its analogues aims at treating anemia caused by specific chronic diseases or by chemotherapy medications that suppress bone marrow activity avoiding the need for RBC transfusion. Other indications for the administration of erythropoiesis stimulating agents (ESAs) can be the anaemia in zidovudine - treated acquired immunodeficiency syndrome (AIDS), the maintenance of RBC in elective non-cardiac and non-vascular surgery, and the increased demands in autologous blood predonation programs for surgical patients[25]. Administration and dosing of rHuEPO are described in Table 1.

Recombinant erythropoietin drugs are known as erythropoietin-stimulating agents (ESAs). There are five erythropoiesis-stimulating agents currently available; epoetin alpha, epoetin beta, epoetin omega, epoetin delta, and darbepoetin alpha. These agents all have the same amino-acid sequence, but glycolysation varies as a result of type- and host cell specific differences in the production process [28].

Epoetin alfa

In 1983, the gene coding for EPO was identified, leading to its synthesis as epoetin alfa by American genetic research corporation, Amgen, who patented the drug under the name Epogen. In 1989, another company, Ortho Biotech, a subsidiary of Johnson and Johnson, began marketing the drug under license as Procrit in the US, and Eprex in the rest of the world. Epoetin alfa is formulated as a colorless liquid in a solution of sodium chloride buffered with sodium citrate or sodium phosphate, and is packaged, for injection, in 1mL vials containing; either 2000, 3000, 4000, or 10,000 International Units (IU) of epoetin-alfa, 5.8 mg sodium citrate, 5.8 mg sodium chloride and 0.06 mg citric acid in water [28].

Epoetin beta

In 1988, the German pharmaceutical company, Boehringer Mannheim (now part of the Roche Group) produced its own recombinant erythropoietin; epoetin-beta, marketed as NeoRecormon. Epoetin beta (Recormon) comes in 1000 IU/0.3mL, 2000 IU/0.3mL, 3000IU/0.3mL, 4000 IU/0.3mL, 5000 IU/0.3mL, 6000 IU/0.3mL, 10,000 IU/0.6mL, and 30,000 IU/0.6mL solutions; and contains urea, sodium chloride, sodium phosphate, and water, in pre-filled syringes for injection. The clinical efficacy of both epoetin alfa and epoitin beta are similar**.**

Darbepoetin alfa

In 2005, Amgen patented a new erythropoietin, darbepoetin alfa, under the brand name Aranesp® Although very similar to EPO, Aranesp®, when administered, has a longer active life than EPO and is approved for use in patients with chronic renal disease, whether or not they are on dialysis.

Epoetin delta

This is one of the newest agents currently available. Called DYNEPO®, this agent is also produced by recombinant technology, from human cell lines. Currently marketed by Shire PLC, it is only available in the European Union. (Sanofi-aventis owns the rights to the product in the U.S., but is not producing it at this time.) DYNEPO® acts like other epoetins and is also indicated for anemia related to chronic kidney disease. It has received considerable attention in the sports world because DYNEPO® resembles human EPO and may not be detected by standard urine tests.

Table 1 Administration and dosing of Epoetin alfa in patients [28]

Condition	Initial dose
ESRD or chronic kidney disease of at least stage 3 (eGFR <60)	EPO 2000–8000 units subcutaneously (SQ) once weekly (dose depending upon patient's weight, severity of anemia, and associated symptoms)
Chemotherapy-induced anemia (currently receiving a course of chemotherapy or have received a course within the past 2 months for non-myeloid, non-erythroid cancer)	EPO 30,000–40,000 units SQ once weekly for4 weeks 1
HIV with symptomatic zidovudine-induced anemia	EPO 100 units/kg IV or SQ 3 times weekly for 8–12 weeks
Myelodysplastic syndrome (MDS)	EPO 40,000 units SQ once weekly for 4 weeks
Chronic hepatitis C under treatment with ribavirin and either interferon alfa or peginterferon alfa	initiate EPO 40,000 units SQ once weekly

Group Health's initial dosing recommendations are more conservative than those of Centers for Medicare and Medicaid Services (CMS). CMS recommendations for epoetin alfa: Initial dose no more than 150 units/kg 3 times weekly or 40,000 units weekly. CMS recommendations for darbepoetin alfa: Initial dose 225 mcg/kg once weekly or 500 mcg every 3 weeks (Group Health non-formulary) [28].

Erythropoietin-mediated tissue protection

EPO, like other glycoproteins, is required recognition by a receptor and is decoded into specific intracellular signaling cascades to exert its biological effects. Hence, the membrane receptor is the key to the physiological actions of circulating EPO. EPO can act only on cells with receptors for EPO and all actions of EPO must be mediated by the receptor. EPO receptors (EPOR) are present on the cell membrane as a homodimer [29]. The binding of a single EPO molecule to the receptor dimer induces a major conformational change in the receptor, bringing the two Janus kinase 2 (JAK2) molecules, that are tethered to the cytoplasmic portion of the receptor, into close position and thereby activating JAK2 by mutual cross-phosphorylation. Activated JAK molecules then induce phosphorylation of tyrosine residues in the cytoplasmic domain of EPOR. These phosphorylated tyrosine residues serve as a docking site attracting various intracellular signaling molecules. These secondary signaling molecules are subsequently activated by JAK2-mediated tyrosine phosphorylation and initiate the downstream signal transduction[4].

EPO exerts an anti-apoptotic effect via three main signaling pathways. Firstly , Jak2-STAT-Bcl-2 pathway, a dominant EPO-dependent antiapoptotic mechanism for hematopoietic cells, also has been implicated in tissue protection. A number of investigators have identified regulation of this pathway, either by induction of antiapoptotic molecules (Bcl-2 and Bcl-X_L) or decrease in proapoptotic molecules (Bax, Bak) [30]. Secondly, EPO-mediated activation of the survival kinases extracellular signal-regulated kinase and Akt are critical in the nervous system. The neuroprotective effect of EPO involves the Ras/mitogen activated protein kinase or the phosphatidylinositol $3'$-kinase pathway and selective inhibitors of mitogen-activated protein kinase and phosphatidylinositol $3'$-kinase block the phosphorylation of extracellular signal-regulated kinases and Akt/ Protein kinase B and prevent the effect of EPO, as has been shown by multiple workers using different model systems in vitro and in vivo [30]. Finally, the nuclear

factor-kB system: Jak2-dependent activation of nuclear factor-ΚB has been proposed to mediate neuroprotection of cortical neurons in vitro as inhibition of these messengers blocked protection [30].

Control of erythropoiesis has been once believed to be the only physiological role of EPO. However, later studies showed that several cells outside the hematopoietic system, such as endothelial cells, neural cells, cardiomyocyte and renal cells [31, 32, 33], also express receptors for EPO. These findings suggest that EPO may also provide cytoprotection in nonerythropoietic cells and thus stimulate an interest in EPO as a novel therapeutic agent for a number of organs.

Protective effect of rHuEPO in brain

The tissue-protective effects of rhEPO have been explored using a multitude of preclinical disease models. In neuronal injury models, One well-examined type of injury in many tissues is that caused by ischemia and ischemia-reperfusion. The first studies have been shown that local cerebral administration of rhEPO prevents ischemia-induced learning disability and neuronal death [34, 35]. Further study also shown that rhEPO administration protect against ischemia and free radical injury [34]. Several in vivo studies have confirmed the beneficial neuroprotective effect of rhEPO. For example, rhEPO (5000 IU/kg i.p. injection), administered either at the time of the middle cerebral artery occlusion or after it, decreased the cerebral infarction by as much as 75% [8,35] Moreover, intravenous administration of rhEPO (350–1000 IU/kg) at reperfusion prevented motor neuron apoptosis and neurological disability induced by a spinal cord ischemic injury [36]. Application of systemic rhEPO following experimental subarachnoid hemorrhage restores the autoregulation of cerebral blood flow, reverses basilar artery vasoconstriction, and enhances neuronal survival and functional recovery [37]. More importantly, a clinical trial on the effect of EPO in the treatment of stroke shows reduction of cerebral infarction and improvement of follow-up and outcome scales in rhEPO-treated patients

receiving 33,000 IU per day for 3 days after stroke [38]. Grasso et al shown that EPO can reverse vasospasm, reducing the basilar artery vasoconstriction [39], potentially through a direct effect on vascular endothelium [40]. EPO also modulates inflammation [8] and recruits stem cells [41]. Moreover, EPO can act directly on neurons, attenuating the production of damaging molecules, such as reactive oxygen species[42] or glutamate-stimulated excitotoxicity [43].

Protective effect of rHuEPO in kidney

In addition to the brain, the tissue-protective effects of rhEPO in kidney injury model has been reported. A number of workers have studied the effects of EPO given prior to or after ischemia-reperfusion injury of the kidney. These studies provide evidence for the renoprotective effect of EPO against acute renal injury. Almost all of these studies have demonstrated that EPO could reduce renal dysfunction and ameliorate histological changes of acute tubular necrosis by stimulation of antiapoptotic molecules and inhibition of proapoptotic molecules [12-13, 44-47]. In general, these studies have used relatively high doses of EPO (300 to 5000 U/kg). Early EPO treatment seems to provide better results than late EPO administration. However, renoprotection is also observed even when EPO is given as late as 6 hours after reperfusion [48]. As only one specific study, Vesey et al. showed that high concentrations of rhEPO (200 IU/ml) completely blocked apoptosis and stimulated proliferation of cultured human proximal tubular cells exposed to 1% oxygen for 16 h, followed by a return to normoxia for 24 h [45]. In model of chronic renal injury, the study has demonstrated that long-term therapy of low-dose EPO (darbepoetin) could reduce mortality, renal dysfunction, and renal scarring in rats with the remnant kidney model (5/6 nephrectomy) [49]. Another report using standard dose darbepoetin similarly provides renoprotection in remnant kidney model, but this occurs in association with increasing hematocrit [50]. Other workers have found EPO beneficial in a wide variety of renal injury models including cisplatin [51, 52] and radiocontrast toxicity [53], ureteral obstruction [54] and diabetic nephropathy [55].

Protective effect of rHuEPO in heart

Many studies in ischaemia/reperfusion animal models of cardiac infarct have been reported. When administered either before the onset of ischemia [56, 57, 58] or at the beginning of reperfusion [59]. EPO reduces the infarct area size, improves recovery of mechanical function and increases coronary flow, partly by exerting anti-apoptotic effects on cardiomyocytes and partly by preventing endothelial cell apoptosis. Chong et al showed that EPO prevents the release of proapoptotic proteins such as cytochrome c and caspase-3 from the mitochondrial membrane by upregulating the phosphoinositide-3 kinases and protein kinase B (PI3 K/Akt) pathway [60] . In addition, a recent study showed that EPO induces the up-regulation of the PI3 K/Akt pathway is associated with the enhanced expression of endothelial nitric oxide synthase (eNOS) in isolated mice cardiomyocytes, which resulted in increased levels of nitric oxide [61]. The administration of EPO to wild-type and $eNOS^{-/-}$ mice before ischemia resulted in increased expression of eNOS and decreased infarct size only in the wild-type mice. The administration of a PI3 kinase inhibitor to isolated cardiomyocytes also completely blocked the antiapoptotic effects of EPO in this study [61]. This further supports the importance of PI3k/Akt signaling in inhibiting apoptosis with EPO. Two studies have shown that EPO administration reduces cardiac dysfunction in doxorubicin-induced cardiomyopathy, partly by increasing endothelial progenitor cells (EPC) numbers and their functional properties [62, 63]. Recently, Westenbrink et al showed that the administration of EPO increased circulating endothelial progenitor cells and improved endothelial function in both ischemic and nonischemic myocardial tissue. However, neovascularization was only observed in ischemic tissue [64]. These observations support the concept that EPO exerts proangiogenic effects by endothelial progenitor cell mediated neovascularization. Moreover, in model of chronic heart injury study has demonstrated that long-term therapy of low-dose EPO (darbepoetin) that given once every three weeks after myocardial infarction, was sufficient to improve cardiac function and induces neovascularisation at a dose that does not increase haematocrit [65].

Protective effect of rHuEPO in liver

The protective effects of EPO in liver injury models have also been reported. The first study was suggested by Yilmez et al. [66], who showed that administration of rHuEPO (1000 IU/kg) 120 min before the onset of the ischemia can reduce liver damage as assessed by histopathological score as well as alanine aminotransferase (ALT) and TNF-α level. The further studies have also shown that administration of rHuEPO 5 min before ischemia can reduce the biochemical evidence of liver injury. However, this protective action was not evident when rHuEPO was administered 5 min before reperfusion. Early administration of rHuEPO was able to reduce oxidative stress and caspase-3 activation, suggesting a subsequent reduction in apoptosis [67, 68]. Administration of EPO 45 min prior to warm ischemia leads to a significant reduction in the levels of alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH), and dramatically reduces apoptosis rates [69]. Madro et al shown that the administration of EPO during galactosamine-induced hepatic damage can decreases gelatinase (MMP-2 and MMP-9) activity, that degrade type IV collagen, a major component of basement membrane [70]. Moreover, the recent study in the animal model of cholestatis fibrosis has demonstrated that EPO treatment significantly diminished the area of focal necrosis, reduced the infiltration of macrophages, decreased levels of profibrotic genes, lowered collagen deposition and reduced systemic anemia caused by bile duct ligation [71].

According to the cytoprotective effects of EPO in various models, to elicit tissue protection, one has to administer exogenous rHuEPO at a relatively high dose which, in turn, will be complicated by pathologic increases in red blood cell mass, i.e. polycythemia. This drawback limits clinical use of rHuEPO for tissue protection; therefore a non-erythropoietic EPO that provides tissue protection is strongly required.

Non-erythropoietic erythropoietin derivatives

Satake R (1990) et al modified EPO molecules with several group-specific reagents in order to study the role of each kind of amino-acid residue in its biological activity. Theresults show that the electric charge on lysine residues of EPO molecule was critical in mediating the erythropoiesis effect [25]. Neutralizing the lysine positive charge, for example, by acetylation, carbamylation or by making the lysine charge negative through succinylation, caused significantly decreased erythropioesis. The next study by Mun KC (2000) et al showed that EPO exposed to high levels of cyanate demonstrates diminished biologic activity in healthy Sprague-Dawley rats. This effect may be manifested by the carbamylation of EPO by the cyanate [72].

Recently, carbamylated EPO (CEPO) has been created by subjecting rHuEPO to cyanide carbamylation (in strict terminology; *carbamoylation)* [17]. This molecule is the first modified rHuEPO to provide tissue protection without significantly increased red blood cell production [17-23]. Further studies suggest that CEPO may not mediate its actions by the conventional homodimerization of EPO receptors (EPOR), but by heterotrimerization between one EPOR and two IL-3 beta common receptors (βCR) [24]. Thus, the carbamylation reacts on free amino acids; eight lysine residues and one terminal amino acid on EPO are potential targets.

As previously described by Satake and colleauges, the modification of the lysine charge negative through carbamylation, acetylation or succinylation caused significantly decreased erythropioesis [25]. The other chemical modifications of lysine residues, for example, phosphopyridoxylation and gluteraldehyde modification are a simple reaction that reduce lysine positive charge of lysine residues; this may also change protein conformation and function [73, 74, 75]. However, whether these lysine modified EPOs retain tissue protective activities like CEPO remains to be elucidated. In addition, it should be possible to obtain novel non-erythropiotic EPOs with therapeutic potential by targeting lysine modification with other methods as well.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Microcentifuge tube : 1.5 ml, 2.0 ml (Elkay, U.S.A.)

1.2 Pipette tip : 10 ul, 100 ul, 1000 ul (Elkay, U.S.A.)

1.3 Microcentrifuge tube : 15 ml, 50 ml (Nunc, U.S.A.)

1.4 Microcon Centrifugal Filter (Millipore, U.S.A.)

1.5 Beaker : 50 ml, 100 ml, 500 ml (Pyrex)

1.6 Flask : 250 ml, 500 ml, 1000 ml (Pyrex)

1.7 Cylinder : 50 ml, 100 ml, 500 ml (Witeg, Germany)

1.8 Petridish : 3.5 cm, 10 cm (Nunc, U.S.A.)

1.9 Well plate : 24 well, 96 well (Nunc, U.S.A.)

1.10 Steriled seropipette : 5 ml, 10 ml (Nunc, U.S.A.)

1.11 PVDF membrane (Amersham, U.S.A.)

1.12 Plastic wrap

1.13 Hyperfilm ECL (Amersham, U.S.A.)

1.14 Glass slide (Sail brand, China)

1.15 Cover slip (Sail brand, China)

2. Equipments

2.1 Stirrer (Bamstead, U.S.A.)

2.2 Vortex (Tommy, Japan)

2.3 Light microscope (Olympus, Japan)

2.4 Autoclave (Thermo, U.S.A.)

2.5 pH meter (Eutech Cybernatics, U.S.A.)

2.6 Balance (Precisa, Switzerland)

2.7 Refrigerated centrifuge (Kubota, Japan)

2.8 Pipette aid (Tecnomara, Switzerland)

2.9 Microwave (Sharp, Japan)

2.10 Thermocycler (Agilent technologies, U.S.A.)

2.11 Water bath (Daihan scientific, Korea)

2.12 CO_2 incubator (Memmert, Germany)

2.13 Spectrophotometer (Biowave II, UK)

2.14 Laminar air flow (Esco, U.S.A.)

2.15 Power supply (Thermo, U.S.A.)

2.16 Heat block (Thermo, U.S.A.)

2.17 Autopipette (Biohit, Finland)

2.18 UV transilluminator (Vilber lourmat, France)

2.19 Gel doc (Tommy, Japan)

2.20 Horizontal gel box (Mupid, Japan)

2.21 Vertical gel box (Apollo, U.S.A.)

2.22 Transfer equipment (Biorad, U.S.A.)

2.23 Reciprocal shaker (Major science, U.S.A.)

2.24 Dimension RXL automated analyzer (Dade behring, U.S.A.)

2.25 Refrigerator (Mitsubishi, Japan)

2.26 Deep freeze (Sanyo, Japan)

2.27 Liquid nitrogen tank (Thermo, U.S.A.)

2.28 Microtome (Thermo, U.S.A.)

2.29 Zetasizer (Malvern Instruments, UK)

3. Reagents

3.1 Cyanate (Sigma)

3.2 Calcium acetate (Sigma)

3.3 Ethylenediaminetetraacetic acid (Sigma)

3.4 Etidium bromide (Sigma)

3.5 Sodium acetate (sigma)

3.6 Triton X-100 (Merck)

3.7 25% Glutaraldehyde solution (Ajax finechem)

3.8 O-Methylisourea (Sigma)

3.9 Sodium chloride (Sigma)

3.10 Sodium hydroxide (Sigma)

3.11 Pyridoxal–5–phosphate (Sigma)

3.12 Sodium borohydride (Sigma)

3.13 N-Acetylbenzotriazole (Sigma)

3.14 rHuEpo (Eprex®) (Janssen-Cilag Thailand)

3.15 Succinic anhydride (Sigma)

3.16 Polyacrylamide solution (Amresco)

3.17 Amnomium hydroxide (Sigma)

3.18 Coomassie Brilliant Blue (Fluka)

3.19 Fetal bovine Serum (Gibco)

3.20 DMEM (Gibco)

3.21 L-Glutamine (Gibco)

3.22 Penicillin G –streptomycin (Gibco)

3.23 Hydrogenperoxide (Siampharmacy)

3.24 Phenylmethylsulfonylfluoride (Sigma)

3.25 Bradford protein assay (BioRad)

3.26 βCR antibody (Santa Cruz Biotechnology)

3.27 EPOR antibody (Santa Cruz Biotechnology)

3.28 Bcl-2 antibody (Santa Cruz Biotechnology)

3.29 ECL system (BioRad)

3.30 Quick Cell Proliferation Assay Kit (Biovision)

3.31 ApopTag (Chemicon)

3.32 Purezol (Biorad)

3.33 Protoscript® first strand cDNA synthesis kit (New England Biolabs)

3.34 *EXTaq* DNA polymerase (Takara)

3.35 Biotinylated secondary antibodies (Vector)

3.36 Avidin peroxidase (Vectastain®)

3.37 3,3' Diaminobenzidine tetrahydrochloride (Dako)

3.38 Trinitrobenzenesulfonic (Sigma)

3.39 Sodium dodecyl sulfate (Sigma)

3.40 Hematoxylin (Sigma)

3.41 Eosin (Sigma)

4. Animals and cell lines

4.1 ICR mice (National Laboratory Animal Center, Nakhon Pathom, Thailand)

4.2 HEK 293 cells (Cell Line Service Eppelheim, Germany)

4.3 unstimulated murine P19 cells (Cell Line Service Eppelheim, Germany)

4.4 UT-7 cells (gift from Nicole Casadevall, Hospital Hotel Dieu, Paris, France)

5. Methods

Pharmaceutical preparation rHuEPO (Eprex®), in prefilled syringe, was purchased from Janssen-Cilag Thailand. According to the company's declaration to Thailand FDA, this preparation consists of rHuEPO alfa 2,000 IU, NaCl 4.38 mg/ml, Glycine 5.0 mg/ml, Polysorbate 80 0.3 mg/ml. The amount of protein concentration in each syringe is 0.03 mg/ml.

Chemical modification of lysine residues on rHuEPO

Carbamylation: 30 µg of rHuEpo was mixed with 2 M cyanate solution to a final concentration of 1 M and the reaction mixture was incubated for 1 day at 37°C [76].

Acetylation: 30 µg of rHuEpo in 100 mM phosphate buffer was mixed with N-acetylbenzotriazole in 30 s to make a final concentration of 9.6 mM [77].

Succinylation: 30 µg of rHuEpo in 0.5 M $NaHCO_3$ (pH 8.0) containing 0.2 M NaCl was incubated with a 15-fold molar excess of succinic anhydride for 1 h at 25°C [78]

Glutaraldehyde modification: 30 µg of rHuEpo in 10 mM sodium acetate and 5 mM calcium acetate buffer (pH 7.5) was mixed with 25% glutaraldehyde solution to make a final concentration of 0.25% glutaraldehyde in mixture solution. The mixture solution was gently stirred for 2 h at 4 °C [79].

Guanidination: rHuEpo 30 µg of was mixed with 200 µL Tris HCl, pH 8.4, containing 1M NaCl. 1000 µl of 1.0 M O-methylisourea was then added to the mixture. The pH was adjusted to 10.5 with NaOH. The mixture was stirred for 4 days at 4°C [80].

Phosphopyridoxylation: rHuEpo was mixed with a 100-fold molar excess of pyridoxal–5–phosphate. Reactions was carried out for 15 min at 25°C. Subsequently, a freshly prepared solution of sodium borohydride (30 mg/ml) was added to the reaction mixture to achieve a final concentration of 0.6 mg/mL [81].

The excess reagents were removed by extensive dialysis against water at 4°C, followed by centrifugal-ultrafiltration with Microcon Centrifugal Filter (Millipore, Bedford, MA, U.S.A.). The modified EPOs from the above reactions was carbamylated EPO (CEPO), acetylated EPO (AEPO), succinylated EPO (SEPO), glutaraldehyde EPO (GEPO) and phosphopyridoxylated EPO (PEPO).

Characterization of lysine residues on rHuEPO

The trinitrobenzenesulfonic (TNBS) acid method

The loss of free lysine residues was determined by using the trinitrobenzenesulfonic (TNBS) acid method as previously described [82]. Fifty microliters of 0.1% trinitrobenzenesulfonic acid was added to 1 ml of each EPO derivative (500 units/ml in normal saline) and 1 ml of 4% sodium hydrogen carbonate (pH 8.4) and this was incubated for 1 h at 37 °C. Absorbance was then measured at 340 nm against a sample blank, and the

trinitrobenzenesulfonic acid reactivity was expressed as a percentage of the absorbance obtained for the nonchemical lysine- modified EPO.

ζ-potential analysis

The magnitude of electrostatic charges on the protein surface will be determined by measuring the ζ-potential of protein dispersion as a function of pHs. ζ-potential values was obtained by using Zetasizer (NanoZS 4700, Malvern Instruments, UK) at room temperature. All measurements were conducted at a wavelength of 633 nm at 25°C with a scattering angle of 90°. The reported results will be the mean of three determinations.

Quantitation of EPO and EPO derivatives Biorad protein assay

Concentration of EPO and EPO derivatives were determined by *Biorad protein assay* , based on the method of Bradford. One part of Dye Reagent Concentrate was diluted with four parts of distilled deionized water, then filtered through Whatman #1 filter to remove particulates. Five dilutions of BSA standard were prepared. 100 µl of each standard and sample solution were added into a clean dry tube. Then, 5.0 ml of diluted dye reagent was added to each tube and vortex. The mixture was incubated at room temperature for at least 5 minutes. Absorbance was measured at 595 nm

Native polyacrylamide gel electrophoresis (PAGE)

The surface charges of EPO derivative was also determined by native PAGE. Twenty micrograms of each EPO derivative was subjected to electrophoresis in 7% native polyacrylamide gel. The composition of separating gel was 30%/0.8% w/v acrylamide/Bis-acrylamide 2.6 ml, 0.375M Tris-HCl pH 8.8 7.29 ml, 10% w/v ammonium persulfate 100 µl and TEMED 100 µl. The running buffer was composed of 25 mM Tris and 192 mM glycine, pH 8.3. To visualize the protein, gels was stained with Coomassie Brilliant Blue.

Evaluation of erythropoietic effect of EPO derivatives

In vitro study; UT-7 colony forming and proliferation assay

The human leukemic UT-7 cell line is growth factor-dependent capable of growing in interleukin-3 (IL-3), granulocyte/macrophage colony-stimulating factor (GM-CSF), or EPO for proliferation and differentiation. The UT-7 cell line is established from marrow cells obtained from a patient with acute megakaryoblastic leukemia. A cell line in which growth is totally dependent on the presence of EPO would provide great help in analysis of the molecular mechanisms by which EPO promotes the growth and differentiation of hematopoietic cells[74].

For colony forming unit (CFU) assay, UT-7cells were cultured in semisolid gel consisting of 10% FBS DMEM, 3% methylcellulose with or without erythropoietin derivative (0.03 μg/ml). The forming colonies was count at day 7 and day 14. For MTT cell proliferation assay, UT-7 cells was cultured in 10% FBS DMEM supplemented with 2mM L-glutamine, 100 units/ml penicillin G -streptomycin with and without erythropoietin derivative supplementation for 2 days. Cell proliferation was measured by using the Quick Cell Proliferation Assay Kit (Biovision,CA, U.S.A.) according to the manufacturer ‘s instruction. In short, the assay was started by adding 100 μl/ml of WST-1/ECS solution to each well. The cells were then incubated for 1 h in culture condition. The measurement of cell proliferation was determined by measuring the absorbance at 440 nm with spectrometer

In vivo study

ICR female mice (30-35 g) were purchased from the National Laboratory Animal Center (Nakhon Pathom, Thailand). They were housed in a temperature-controlled facility with a 12 h light on-off schedule, and free access to diet and water. All procedures involving animals were performed according to the animal care and ethics legislation of Lerdsin General Hospital. Five mice were injected with 0.3 mg/kg of each EPO derivative on days 0, 3, 7, 10 and 12. Hematocrit

was measured from tail blood on days 0, 7 and 14, briefly, mice blood in capillary tube was centrifuged at 10,000 rpm for five minutes. Blood was separated into two layers ; packed red cells and plasma. Hematocrit percentage = (100/total sample length)x packed red cell lengh

Evaluation of tissue protective of EPO derivatives

In vitro study

Oxidative stress and serum deprivation induced cell damage

HEK-293 cells (Cell Line Service Eppelheim, Germany) were cultured in 10% FBS DMEM (Gibco, Grand Island, NY, U.S.A.) supplemented with 2 mM L-glutamine, 100 units/ml penicillin G -streptomycin. For oxidation-induced cell damage, HEK-293 cells were cultured overnight with serum-free medium or pretreated with rHuEPO derivatives in serum-free medium to a final concentration of 0.03 μg/ml. To induce apoptosis, the culture media was replaced with freshly prepared culture media supplement with 2mM H_2O_2 for 2 h.

For serum deprivation-induced cell damage, P19 cells were cultured overnight in media either with or without EPO derivatives. To induce apoptosis, culture media was replaced with serum free media for 2 h [83].

Measurement of LDH concentration

LDH concentration in culture media and lysed cells were measured in triplicate using the Dimension RXL automated analyzer (Dade Behring, Newark, DE, U.S.A.). The percentage of LDH release from cells was determined by calculating the ratio of LDH concentration in culture media to that in lysed cells.

Apoptosis study

Apoptosis on a cell monolayer was detected by in situ DNA fragmentation, using ApopTag (Chemicon, Temecula, CA, U.S.A.), according to the manufacturer's instruction.

Briefly, cells were fixed in cooled ethanol : acetic acid (2:1) for 5 min at -20^{o}c , then washed in PBS twice for 5 min each. Equilibration buffer (13 µl per cm ^{2}) was applied to cells. A working strength TdT enzyme was immediately added and incubated in humidified chamber at 37 ^{o}c for 1 h. Cells were incubated in working strength stop/ wash buffer for 10 min and washed in PBS 3 times for 1 min each. Anti-digoxigenin conjugate (13 µl per cm ^{2}) was applied and incubated in dark humidified chamber at RT for 30 min. Cells were wash washed in PBS 3 times for 2 min each. Color was developed with 3,3' diaminobenzidine tetrahydrochloride. Apoptotic cells were counted as TUNEL positive cells on 15 randomly selected 200x fields or entire specific area. All the scorings were determined in a blinded manner.

Immunoblotting and immunoprecipitation

Cultured cells were lysed in ice-cold RIPA buffer containing 10 mmol/L phenylmethylsulfonylfluoride (PMSF). The supernatant was collected, and the protein concentration was measured using the Bradford protein assay (Biorad, Hercules, CA, U.S.A.). For immunoblotting, 50 µg of protein was loaded onto polyacrylamide gel for electrophoresis under reducing conditions. Separated proteins were then semi- dry transferred to PVDF membrane. For immunoblotting, Membrane were blocked with 3% BSA in PBS, 0.1%Tween 20 for 30 min and incubated with 1:200 βCR antibody in 1%BSA in PBS, 0.1%Tween 20 (K17, Santa Cruz Biotechnology, Santa Cruz, CA), EPOR (M20 and H194, Santa Cruz Biotechnology) at final dilution 1:500 or EPOR 1:500 for 1 h. After the blots were extensively washed and incubated with suitable peroxidase secondary antibodies, the specific protein bands were visualized using the ECL system.

For immunoprecipitation, P19 cells were incubated with culture media supplemented with 0.03 µg/ml GEPO for 20 min. A complex of GEPO-associated receptors were subjected to protein A immunoprecipitation with a mixture of two anti-EPOR antibodies (M20 and H194) at

final dilution 1:200. The precipitation proteins were then electrophoresed and immunoblotted with βCR and EPO antibodies.

In vivo study

Ischemia /reperfusion(I/R) induced kidney injury

ICR mice were subcutaneously injected with rHuEPO derivative (at dose 0.3 mg/kg) or an equal volume of 0.9%NaCl (NSS) (n= 5 per group). Thirty minutes later, under diethyl ether anesthetization, animals were subjected to bilateral I/R injury by simultaneous clamping of both renal pedicles for 40 min. During the ischemic phase, the abdomen was partially closed and the surgical table temperature was set at 39°C as previously described [84]. At 24 h of reperfusion, the animals were sacrificed and the kidneys were removed.

Serum creatinine (SCr) measurement

Blood samples were collected by heart puncture just before sacrifice. SCr was measured using the Dimension RXL automated analyzer.

Apoptotic study

Apoptosis in a I/R kidney cells was detected by in situ DNA fragmentation, using ApopTag according to the manufacturer's instruction based on previous described. Apoptotic cells were counted as TUNEL positive cells on 15 randomly selected 200x fields or entire specific area.

Immunohistochemistry

Immunohistochemistry of Bcl-2 was performed on 3 microns of paraffin embedded, Methyl Carnoy's fixed renal tissues. Endogenous peroxidase activity was quenched by incubation with 3%H_2O_2 in PBS for 10 min. Non-specific binding was blocked with 4% skim milk and 1%BSA in PBS, 0.1%Tween 20 for 30 min. Slide was incubated with 1:500 monoclonal mouse

anti Bcl-2 in 1%BSA in PBS, 0.1%Tween 20 for one hour at room temperature, followed by biotinylated secondary antibodies for 30 min. Avidin peroxidase was applied at the final step, and color was developed with 3,3' diaminobenzidine tetrahydrochloride . Finally, tissues were counterstained with 0.1% hematoxylin. All histological slides were examined by light microscopy.

RT-PCR

Total RNA was isolated from liquid nitrogen snapped tissue with Purezol (Biorad) according to the manufacturer's protocol. 1 ml of Purezol reagent was dropped into 100 mg of tissue ,then this was homogenized for 30-60 sec. The lysate was incubated for 5 min at room temperature. The lysate was added with 0.2 ml chloroform and shaked vigorously for 15 min. The mixture was incubated for 5 min at room temperature and then centrifuged at 12,000xg for 15 min at 4 ^{o}C. The aqueous phase was immediately transferred to a new RNase-free tube. RNA was precipitated by adding 0.5 ml of isopropyl alcohol and centrifugation at 12,000xg for 10 min at 4 ^{o}C. RNA was appeared as a white pellet on the side and bottom of the tube. cDNA synthesis was carried out with 1 μg of isolated RNA using the Protoscript® first strand cDNA synthesis kit (New England Biolabs, Ipswich, MA, U.S.A.). One microgram of cDNA was used for PCR with the corresponding primers Bcl-2: 5'-TGCACCTGACGCCCTTCAC-3', 5'-AGACAGCCAGGAGAAATCAAACAG-3' (product size 245 base pairs) and β-actin: 5'-CTTTCTACAATGAGCTGCGTG-3', 5'-TCATGAGGTAGTCTGTCAGG-3' (product size 356 base pairs) with Stratagene Mx 3000p and *EXTaq* DNA polymerase (Takara, Japan). The PCR products were electrophoresed and visualized under UV light.

Data analysis

Data were expressed as mean ± SD. Multiple comparisons among groups were performed by one-way ANOVA using the post-hoc LSD test (SPSS version 13). $p<0.05$ was considered statistically significant.

CHAPTER IV

RESULTS

Characterization of lysine residues on modified rHuEPOs

TNBS assay

Analysis of free amino acid loss by TNBS assay showed diverse yields of rHuEPO modification in various reactions. More than 90% of free amino acids were lost by gluteraldehyde modification, resulting in gluteraldehyde EPO (GEPO), or by guanidinate reaction, resulting in guanidinate EPO (GuEPO). This was considered highly efficient. The carbamylated reaction (CEPO) resulted in approximately 80% free amino acid loss, while phosphopyrated, acetylated and succinilated reactions reduced free amino acid by only 45.6%, 42.7% and 39.4%, respectively as depicted in Figure 1. Based on these findings, the glutaraldehyde modification and guanidination were considered as the most efficient reactions.

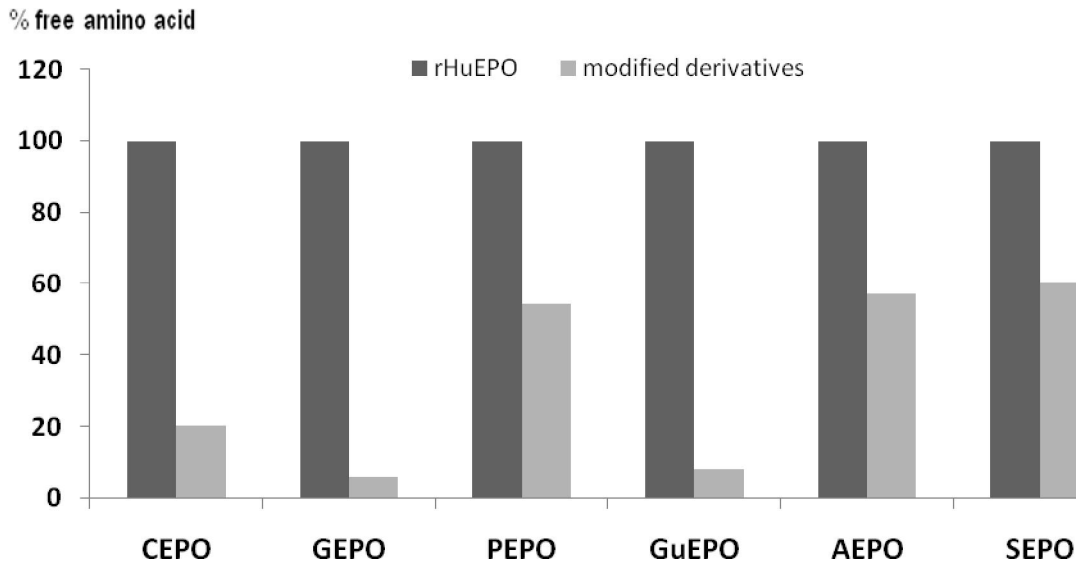


Figure 1: Analysis of free amino acid loss of modified rHuEPOs by TNBS assay showed diverse yields of rHuEPO modification in various reactions.

ζ-potential analysis

To provide further insights into the basic properties of the modified rHuEPO molecules, the surface charge of modified rHuEPO proteins was studied through a zeta-potential measurement. Isoelectric points (ISP), the pH values where the surface electric charge is zero, were also determined. The ISP of rHuEpo was 6.84. GEPO was the most negative with an ISP of 6.00, while, CEPO, PEPO, AEPO and SEPO were slightly negative as compared with ISP of 6.49, 6.53, 6.64 and 6.69 respectively, as compared to the pro drug. On the other hand, GuEPO was more slightly positive than rHuEPO with ISP of 7.12 as shown in table 2

Table 2: Zeta potential analysis of modified rHuEPOs

EPO dervatives	**pH at ISP (at 0 mV)**
EPO	6.84
CEPO	6.49
GEPO	6.00
PEPO	6.53
GuEPO	7.12
AEPO	6.64
SEPO	6.69

Native polyacrylamide gel electrophoresis (PAGE) of EPO, GEPO and GuEPO

According to the results shown above, the further determination of surface electric charge of GEPO and GuEPO was also ascertained by native PAGE . The results showed that GEPO was moved toward the cathode faster than rHuEPO (Figure 2a). In contrast to GEPO, GuEPO was moved toward the cathode slower than rHuEPO (Figure 2b). The results of native PAGE and zeta potential analysis suggested that glutaraldehyde modification caused slightly decrease of positive charge on erythropoietin molecule.

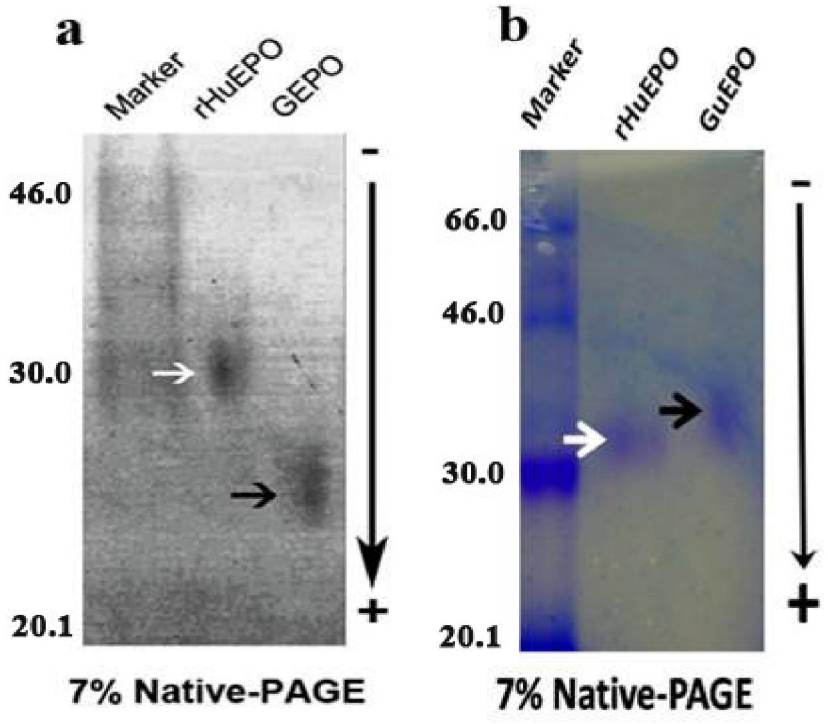


Figure 2: Native PAGE of GEPO and GuEPO compared with EPO

a) The single band of GEPO on native PAGE moved toward the cathode faster than rHuEPO
b) The single band of GuEPO on native PAGE moved toward the cathode slower than rHuEPO

Evaluation of erythropoietic effect of EPO derivatives

In vivo erythropoiesis was performed to test the erythropoietic effect of modified EPOs. Mice were subcutaneously injected with 0.3 mg/kg of rHuEpo or modified-EPOs at days 0, 3, 7, 10 and 12. There were no differences in hematocrit at the beginning, but hematocrit levels were significantly raised in mice receiving all EPOs except for GEPO, by the end of the first week. At day 14, only in mice treated with GEPO were the hematocrit values unchanged and comparable to those of control animals. Interestingly, the hematocrit of the GuEPO-treated group was increased in a similar fashion to that of rHuEPO-treated mice (Figure 3), although most of the free amino acids were drastically modified. Since we were unable to completely modify lysine residues on CEPO, PEPO and SEPO, elevated hematocrit levels in mice treated with these derivatives may be confounded by unmodified EPO and do not necessarily indicate that these derivatives were erythropoietic.

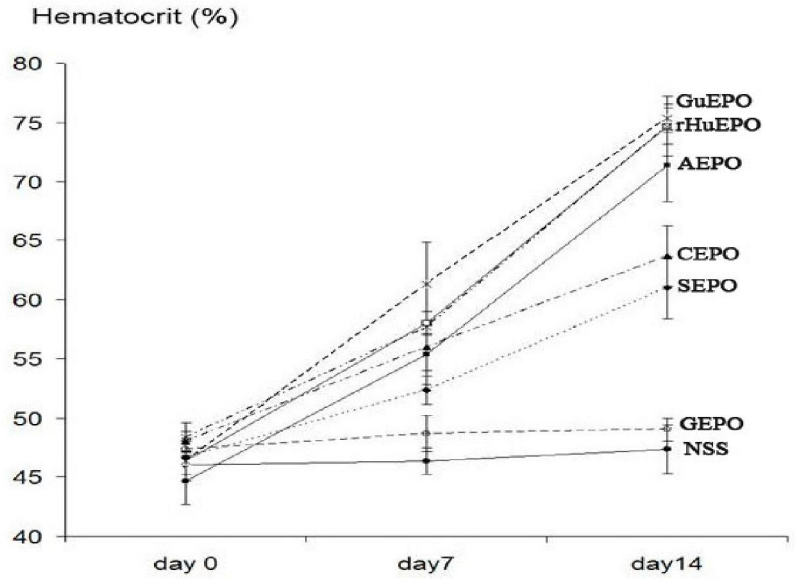


Figure 3: Effect of rHuEPO and modified EPOs on *in vivo* erythropoiesis. rHuEPO and modified EPOs at the doses of 0.3mg/kg were subcutaneously injected in mice The results showed that GEPO was completely devoid of erythropoietic activities.

To confirm that GEPO had no erythropoietic activities, the colony forming of erythopoetic cell lines UT-7 was performed. The colony count of rHuEPO treated UT-7 cells at day 7 and day 14 was 21.3+0.6 and 86.0+4.0. This was significantly higher than that of untreated cells (*=p<0.05 vs. untreated cells, ŧ=p<0.05 vs. GEPO treated cells). In contrast, the colony count of GEPO treated UT-7 was not different from the untreated cells (Figure 4a). MTT proliferation assay also supported this result (Figure 4b) (*=p<0.05 vs. untreated cells, ŧ=p<0.05 vs. GEPO treated cells), and indicated that GEPO was completely loss *in vitro* erythropoiesis. Notably, the proliferation rate of UT-7 cells, pre-saturated with GEPO, was increased by adding rHuEPO to culture media. Since UT-7 proliferation was mediated by homodimeric EPOR, this result suggested that GEPO was not a competitive antagonist of rHuEPO on classical EPO receptors.

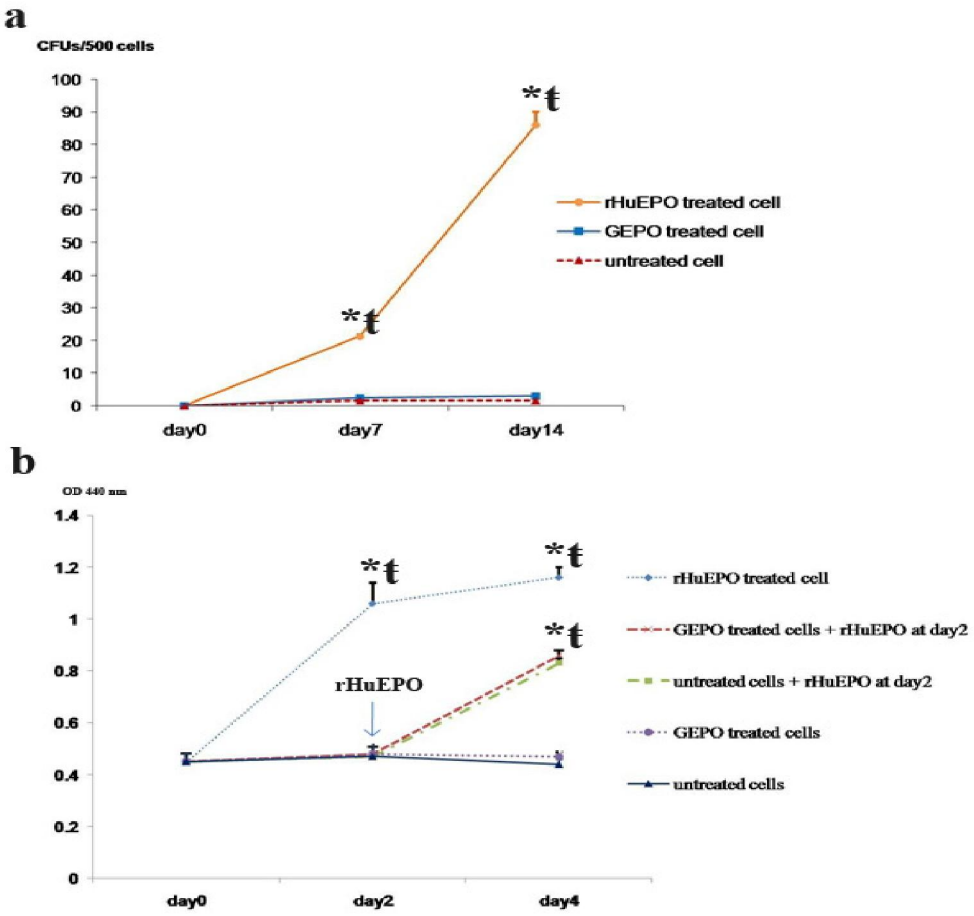


Figure 4: The erythropoietic effect of rHuEPO and GEPO on UT-7 cells

a) The colony count of rHuEPO treated UT-7 cells at day 7 and day 14 was significantly higher than that of GEPO treated and untreated cells. In contrast, the colony count of GEPO treated UT-7 was not differ to the untreated cells. (*=$p<0.05$ vs. untreated cells, ŧ=$p<0.05$ vs. GEPO treated cells)

b) MTT proliferation assay results after culturing UT-7 cells in 10% FBS DMEM supplemented with and without either rHuEPO or GEPO also showed that the proliferation rate of rHuEPO treated UT-7 cells was markedly higher than that of GEPO treated and untreated cells . Notably, the proliferation rate of UT-7 that pre-saturated with GEPO was increased by adding rHuEPO to culture media. (*=$p<0.05$ vs. untreated cells, ŧ=$p<0.05$ vs. GEPO treated cells)

Evaluation of tissue protective of EPO derivatives

In vitro study* : *Oxidative stress and serum deprivation induced cell damage

Measurement of LDH concentration

Cytoprotection of modified EPOs was assessed by determining the ratio of LDH released from HEK-293 cells subjected to oxidative damage and of P19 cells following 2h of serum free culture. LDH release from cells after induced oxidative cell injuries demonstrated that each modified EPO has different cytoprotective activities. The LDH release of H_2O_2-treated HEK-293 cells was 57.23%, which was significantly increased as compared with the non-treated cells and blank media (data not shown). Pretreatment with rHuEPO and modified EPOs significantly reduced LDH. (*=$p<0.05$ vs. control, ŧ=$p<0.05$ vs. rHuEPO) (Figure 5a)

LDH release from P19 cells subjected to serum deprivation is an index of cell damage. The LDH release was highest in the control cells. Pretreatment with EPO derivatives significantly reduced LDH release, suggesting the cytoprotective properties of those molecules. (*=$p<0.05$ vs. control, ŧ=$p<0.05$ vs. rHuEPO) (Figure 5b)

As shown in Figure 5a and b, LDH release was significantly reduced in both cell lines by treatment with EPO and modified derivatives, suggesting that these derivatives retain the cytoprotective activities of EPO.

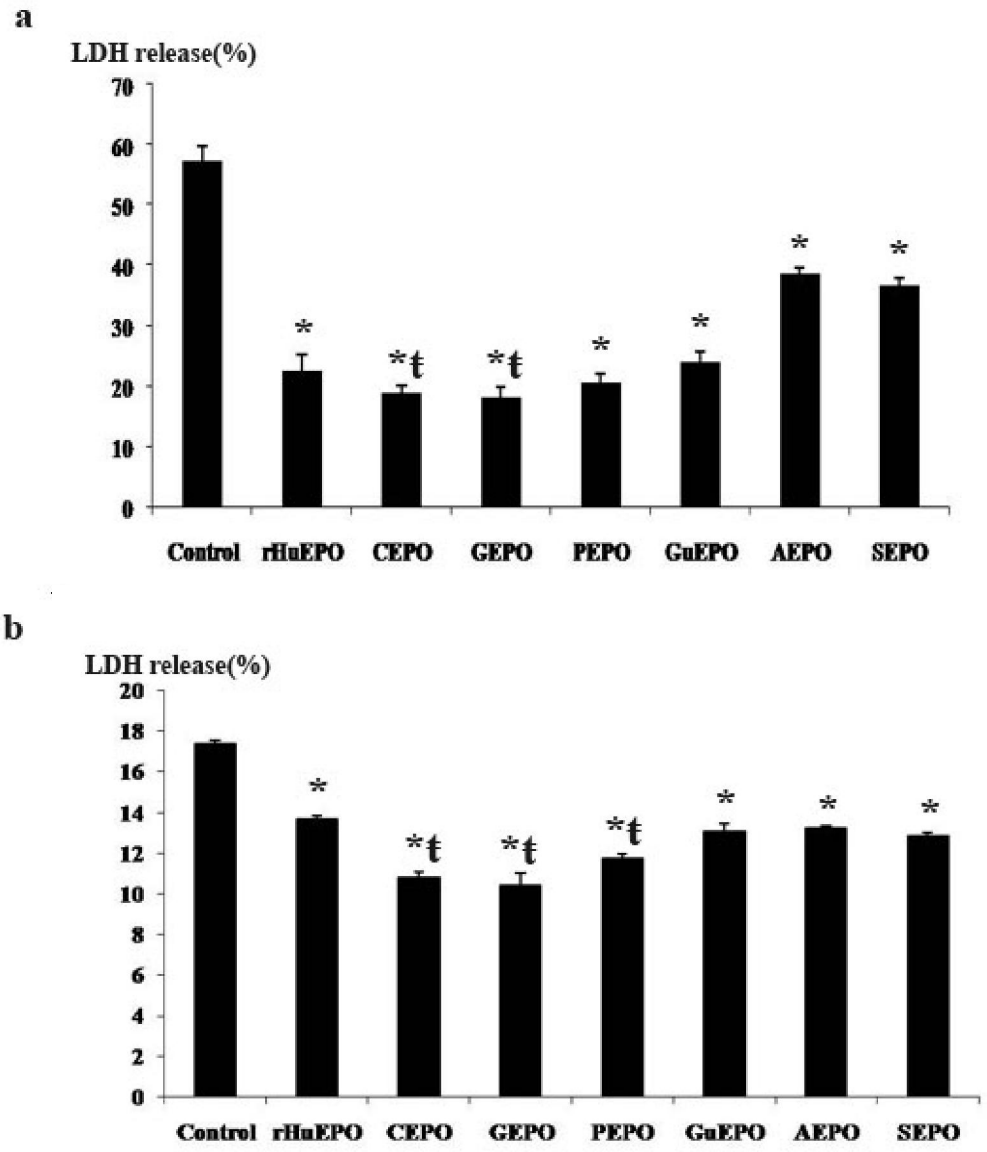


Figure 5: LDH released from cells after inducing cell damage

a) LDH release from cells after induced oxidative cell injuries demonstrated that each modified EPO has different cytoprotective activities. The LDH release of H_2O_2-treated

HEK-293 cells was 57.23%, which was significantly increased as compared with the non-treated cells and blank media (data not shown). Pretreatment with rHuEPO and modified EPOs significantly reduced LDH. (*=$p<0.05$ vs. control, ŧ=$p<0.05$ vs. rHuEPO)

b) LDH release from P19 cells subjected to serum deprivation is an index of cell damage. The LDH release was highest in the control cells. Pretreatment with EPO derivatives significantly reduced LDH release, suggesting the cytoprotective properties of those molecules. (*=$p<0.05$ vs. control, ŧ=$p<0.05$ vs. rHuEPO)

Apoptosis study

The anti-apoptotic effect of rHuEPO and the non-erythropoietic derivatives were tested on both HEK-293 and P19 cell lines. As shown in Figure 6a, the number of TUNEL-positive cells in HEK-293 cells subjected to H_2O_2 (data pooled from three independent experiments) was 60.3$\pm$6.1/5000 cells. Pretreatment with rHuEPO and GEPO significantly decreased the apoptotic cells to 45.3$\pm$3.5 and 27$\pm$4.3 cells/5,000 cells, respectively (* = $p<0.05$). Treatment with CEPO also reduced the number of apoptotic cells, but this trend did not reach a statistically significant level. P19 cells subjected to serum-free media also displayed increased apoptosis (Figure 6b). Pretreatment with rHuEPO and non-erythropoietic derivatives significantly reduced P19 cell apoptosis (* = $p<0.05$). Notably, the number of apoptotic cells was lowest with GEPO treatment. Taken together, these results confirmed that GEPO had anti-apoptotic activities greater than those of the parent drug.

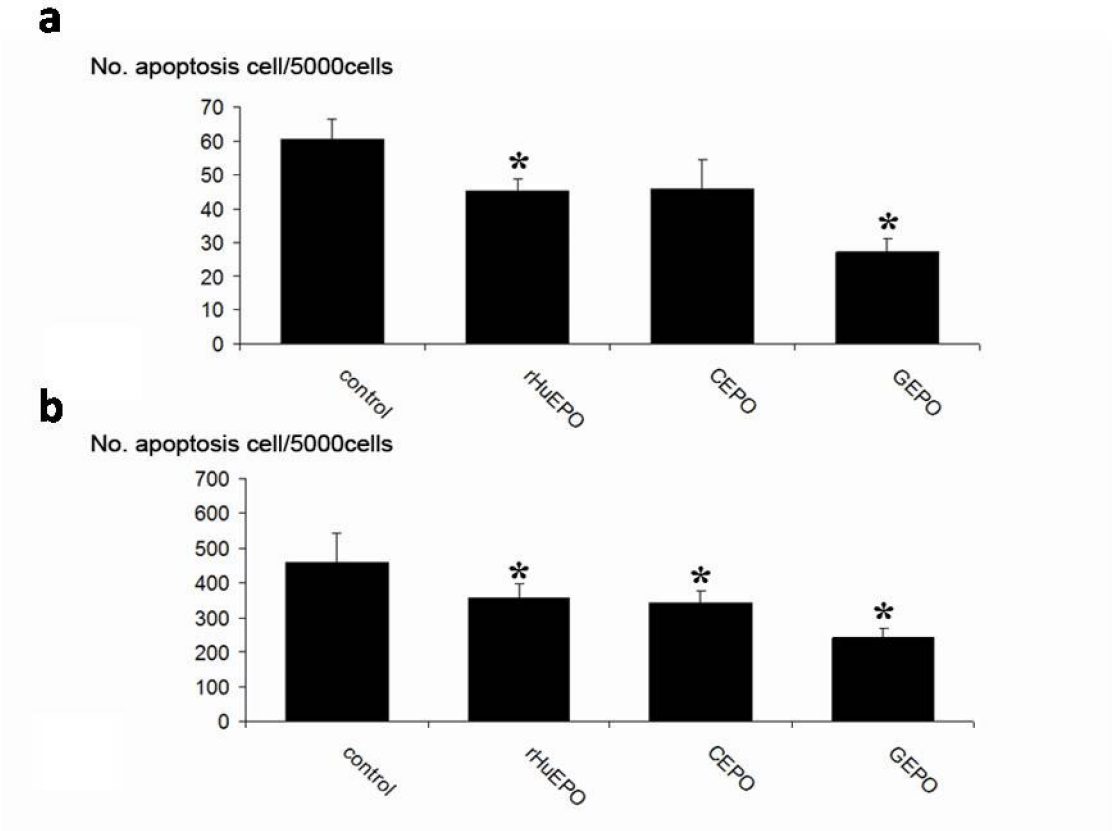


Figure 6: The number of apoptotic cell from cells subjected to oxidative damage.

a) The number of TUNEL-positive cells among cultured HEK-293 cells that were pretreated with modified EPOs demonstrate levels of induced apoptosis. Data from three independent experiments show markedly decreased apoptosis after GEPO pretreatment. (*=$p<0.05$ vs. control).

b) The number of TUNEL-positive P19 cells after 2h serum-free culture of untreated (control) cells and cells treated with modified EPOs. (*=$p<0.05$ vs. control).

Immunoblotting and immunoprecipitation

Since GEPO did not increase red blood cell mass and exhibited cyto-protective effects *in vitro*, we reasoned that GEPO is a novel non-erythopoietic EPO that provides tissue protection. Immunoblotting showed both cell lines expressed EPOR and βCR (Figure 7a). To investigate the binding of GEPO with βCR/EPOR heterotrimeric receptors, immunoprecipitation of cell lysate was performed with EPOR antibodies. Immunoblots of precipitate proteins stained for both βCR and GEPO (Figure 7b), suggested that GEPO bound to the heterotrimeric receptor was composed of EPOR and βCR.

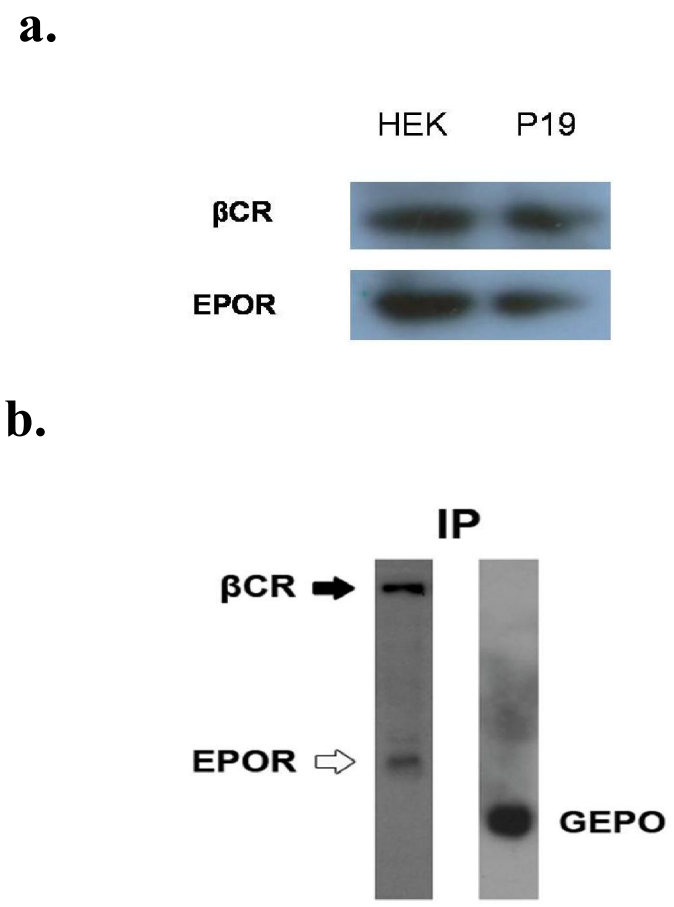


Figure 7: Immunoblot of HEK-293 and P19 cells

a) Immunoblot of HEK-293 and P19 cells showed that both cell lines expressed EPOR and βCR.

b) Lysate proteins from GEPO-treated P19 cells were precipitated with anti-EPOR. Left panel: The precipitate protein was reacted with βCR antibody (arrow) and EPOR antibodies (open arrow). Right panel: The precipitated protein was also detected with anti-EPO antibody with cross reactivity for GEPO.

In vivo study :Ischemia /reperfusion(I/R) induced kidney injury

Serum creatinine (SCr) measurement

I/R induced kidney damage was employed to clarify whether GEPO retained dramatic protective properties when compared to rHuEPO and CEPO. Renal function was assessed by measuring serum creatinine (SCr) at 24 h of reperfusion. In the saline-treated control animals (NSS), SCr was 67.47±7.69 μmol/L. Treatment with GEPO remarkably decreased SCr to 26.40±1.52 umol/L. rHuEPO and CEPO also significantly lessened SCr but to a lesser extent. Levels were 57.79±11.95 and 43.12±6.16 μmol/L, respectively (Figure 8).

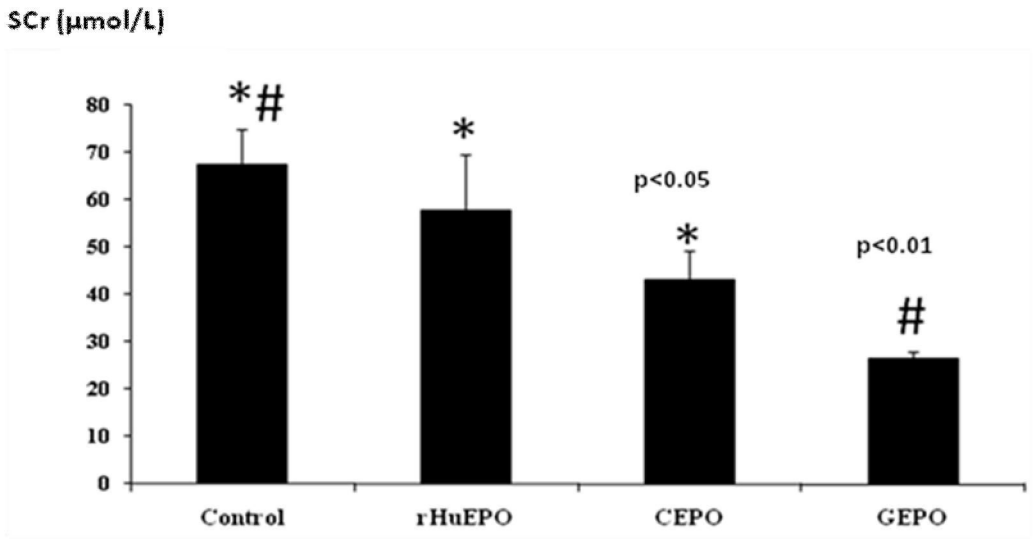


Figure 8: SCr level of I/R kidney injury mice treated with EPO, CEPO and GEPO. Treatment with GEPO significantly decreased SCr to 26.40±1.52 umol/L (#=p<0.01 vs. control). rHuEPO

and CEPO also significantly reduced SCr. Levels were 57.79±11.95 and 43.12±6.16 μmol/L (*=p<0.05 vs. control), respectively

Apoptosis study

A TUNEL assay also demonstrated severe tubular cell apoptosis in the kidneys of the control animals. rHuEPO, GEPO and CEPO were able to inhibit tubular cell apoptosis induced by I/R. The mean number of TUNEL positive cells in the control animals was 86.8±15.8 cells/field. This number decreased to 57.3±26.7, 52.3±28.06 and 23.8±13.9 cells/field for rHuEPO, CEPO and GEPO, respectively. (Figure 9)

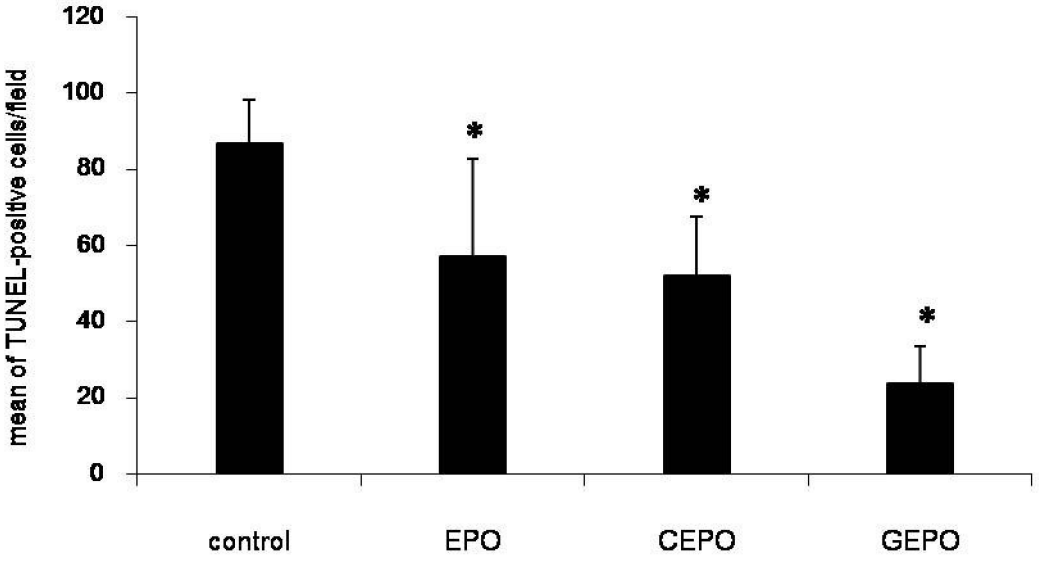
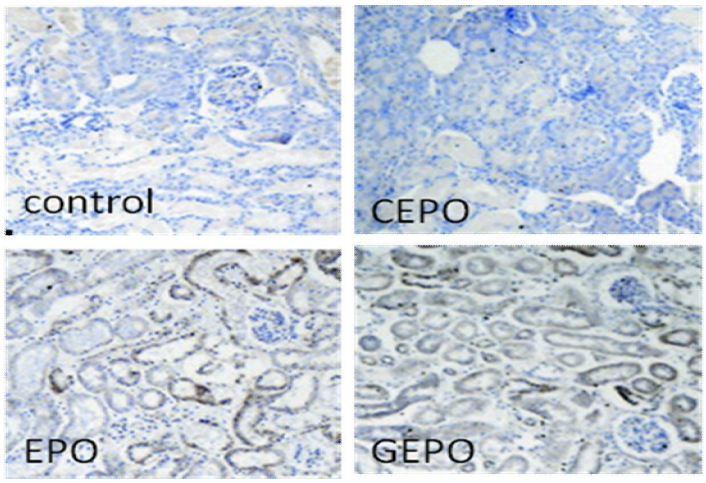


Figure 9: The number of apoptotic cell in the I/R kidneys of saline, EPO, CEPO and GEPO-treated mice. A TUNEL assay showed prominent apoptosis of cells in the kidneys of saline-treated animals. The numbers of apoptotic cells were significantly decreased in GEPO- rHuEPO- and CEPO- treated mice (*=p<0.05 vs. control)

Immunohistochemistry and RT-PCR

Immunohistochemistry analysis showed that rHuEPO- and GEPO-treated animals expressed higher Bcl-2 protein in renal tissues as compared to the control and CEPO-treated animal (Figure 10a). This result was also confirmed by RT-PCR of Bcl-2. RT-PCR also showed markedly increased intra-renal Bcl-2 transcription in rHuEPO-and GEPO -treated animals as compared to saline-treated animals. Bcl-2 transcription was slightly increased in CEPO-treated mice. (Figure 10b)

a



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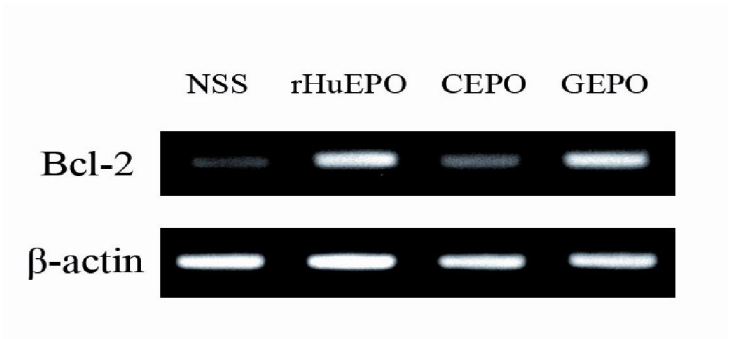

Figure 10 : Immunohistochemistry analysis and RT-PCR of Bcl-2 in kidney of II/R mice.

a) Immunohistochemistry analysis demonstrated rHuEPO- and GEPO- treated mice expressed higher Bcl-2 protein in renal tissues as compare to the control and CEPO-treated mice.

b) RT-PCR also showed markedly increased intra-renal Bcl-2 transcription in rHuEPO-and GEPO -treated animals as compared to saline-treated animals. Bcl-2 transcription was slightly increased in CEPO-treated mice.

CHAPTER V

DISCUSSION

This current work reports simple reactions to modify lysine residues on the marketed formulation of rHuEPO in order to obtain non-erythropoietic EPO. The formulation that we employed contained polysorbate 80 and glycine as stabilizers in a fixed ratio that was stoichiometric with EPO [85]. Previous works indicated that this preparation formed polysorbate 80-EPO-associated micelles [86]. On account of the complexity of the formulation, the effectiveness of modifications was low and discrepant to the previous works especially in carbamylated [17, 25], phosphorylated and succinated reactions [25]. In contrast, the glutaraldehyde modification and guanidination were considerably more effective on this rHuEPO formulation. Cytotoxicity screening and *in vivo* administration revealed that GEPO lost its erythropoietic effect, but still had cytopotective activity; it was therefore considered to be a non-erythropoietic EPO. Notably, SEPO also shared some properties of the non-erythropoietic EPO. However, because of the incomplete modifications we were unable to demonstrate this conclusively and further elucidate the molecule's actions.

Satake and colleagues speculated that lysine residues on the erythropoietin molecule, specifically their positive charge, were crucial in mediating the erythropoietic effect. In their work, decreasing lysines' positive charge reduced erythropoietic activities, while chemical reactions which unchanged lysines' positive charge, such as guanidination retained the *in vitro* erythropoietic activities. Our *in vivo* experiment clearly showed that GuEPO induced erythropoiesis. The results of native PAGE and zeta potential analysis suggested that guanidination caused slightly increase of positive charge on erythropoietin molecule. Satake previously reported that GuEPO increased red blood cell production than the pro-drug. Therefore, our results confirmed Satake's *in vitro* finding.

Gluteraldehyde modification is a simple reaction that reduces lysine positive charge by inducing a crosslink at lysine residues. This may also change protein conformation and function [73, 74]. Theoretically, the gluteraldehyde reaction may be able to induce lysine crosslinks between two or more molecules of rHuEPO. However, a single band of GEPO on native-PAGE suggested that the reaction induced intra-molecular crosslinks rather than inter-molecular

crosslinks. The native PAGE and zeta potential analysis confirmed that GEPO was more negative than rHuEPO supporting the previous notion that positive lysine charge on EPO is essential for erythropoiesis.

Previous studies indicated that cytoprotection of rHuEPO and CEPO were mediated, at least in part, by their anti-apoptotic activity [18, 19]. In this work, we test the anti-apoptotic activity of GEPO with two unrelated apoptotic models, H_2O_2 in HEK cells and serum deprivation in P19 cells. H_2O_2 induced apoptosis is a representative model of reactive oxygen species (ROS) induces apoptosis [87, 88, 89]. The serum deprivation of P19 cells is also a standard apoptogenic model and represent for the loss of surviving signal induces apoptosis [90, 91].

Studies from Brines and Cerami suggest that CEPO binds to a βCR/EPOR heterotrimeric receptor, unlike EPO, which binds to homodimeric EPOR. Therefore, differential receptor binding was postulated to be the underlying mechanism that dissociates cytoprotective actions of EPO from its erythropoietic actions. Nonetheless, this postulate is difficult to verify as a finding that is universally true; it may be a CEPO-specific observation if the only available non-erythropioetic EPO was CEPO. Recent works also showed the piece of evidences that EPO might directly interact with βCR. This work demonstrates that GEPO, the other non-erythropoietic EPO, also interacted with heterotrimeric receptors. Moreover, the data on UT-7 suggested that GEPO was not a competitive antagonist of EPO on erythropoietic proliferation thereby strengthening evidence that differential receptor binding controlles EPO actions. However, the action of GEPO was not identical to CEPO, at least with regard to Bcl-2 activation. Evidence suggests that tissues protection mediated by EPO was also related to Bcl-2 activation [4, 12]. However, CEPO did not activate Bcl-2 in a previous study [17]. The present work found that GEPO, similar to rHuEPO, was able to activate Bcl-2. Taken together, these results suggest that GEPO cytoprotective action is similar to that of EPO, though this molecule was free of erythropoietic activity.

Previous studies were able to demonstrate a renoprotective action of rHuEPO in I/R-induced kidney damage, via multiple mechanisms [12, 13, 45, 46, 92, 93]. Yang's group showed that the kidney protection of EPO is associated with HSP70 activation. A study by Patel provided evidence that rHuEPO reduced intra-renal inflammation, as shown by decreased renal malondialdehyde [91]. Gong and coworkers showed that rHuEpo prevented the down- regulation

of sodium channels and aquaporin, the proper functioning of which is essential for maintaining renal functions. Recently, the renoprotection of CEPO was demonstrated in the context of I/R with decreasing apoptosis as an index [22]. This current work clearly demonstrates that the novel non-erythropoietc EPO, GEPO, also provided substantial renoprotection. Pretreatment with GEPO significantly decreased histological damage and preserved renal function, accompanied by increased intra-renal Bcl-2 transcription. GEPO may exert renoprotective action in the context of I/R induced renal damage by inhibiting apoptosis via the Bcl-2 pathway. In addition to I/R induced kidney damage, renoprotection without erythropoietic activity of GEPO may have therapeutic advantage for long term use in chronic evolving renal diseases for example diabetic renal disease [94] and secondary renal progression [49].

In conclusion, GEPO, the novel non-erythropoietic EPO described here was efficiently obtained from a simple reaction of commercial rHuEPO. This series of experiments showed that this agent possesses potent cytoprotective properties both *in vitro* and *in vivo*, with promise for future use as an adjunctive treatment for kidney disease.

REFERENCES

[1] Lacombe, C., Mayeux, P. Biological of erythropoietin. Haematologica. 83 (1998) : 724-732.

[2] Lacombe, C., Mayeux, P. The molecular biological of erythropoietin. Nephrol Dial Transplant. 14 (1999) : 22-28.

[3] Fisher, J.W. Erythropoietin: Physiology and Pharmacology Update. Exp Biol Med. 228 (2003) : 1-14.

[4] Brines, M., Cerami, A. Discovering erythropoietin's extra-hematopoietic functions: Biological and clinical promise. Kidney Int. 70 (2006) : 246-250.

[5] Marti, H.H., Wenger, R.H., Rivas, .LA., Straumann, U., Digicaylioglu, M., Henn, V., et al. Erythropoietin gene expression in human, monkey and murine brain. Eur J Neurosci. 8 (1996) : 666-676.

[6] Morishita, E., Masuda, S., Nagao, M., Yasuda, Y., Sasaki, R., et al. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. Neuroscience.76 (1997) : 105-116.

[7] Maiese, K., Li, F., Chong, Z.Z. New avenues of exploration for erythropoietin. JAMA. 293 (2005) : 90-95.

[8] Brines, M.L., Ghezzi, P., Keenan, S., Agnello, D., de Lanerolle, N.C., Cerami, C. et al. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci U S A. 97 (2000) : 10526-10531.

[9] Gorio, A., Gokmen, N., Erbayraktar, S., Yilmaz, O., Madaschi, L., Cichetti, C. et al. Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. Proc Natl Acad Sci U S A. 99 (2002) : 9450-9455.

[10] Junk, A.K., Mammis, A., Savitz, S.I., Singh, M., Roth, S., Malhotra, S., et al. Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. Proc Natl Acad Sci USA 99 (2002) : 10659- 10664.

[11] Calvillo, L., Latini, R., Kajstura, J., Leri, A., Anversa, P., Ghezzi, P., et al. Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. Proc Natl Acad Sci U S A.100 (2003) : 4802-4806.

[12] Yang, C.W., Li, C., Jung, J.Y., Shin, S.J., Choi, B.S., Lim ,S.W. et al. Preconditioning with erythropoietin protects against subsequent ischemia-reperfusion injury in rat kidney. FASEB J. 17 (2003) : 1754-1755.

[13] Abdelrahman, M., Sharples, E.J., McDonald, M.C., Collin, M., Patel, N.S., Yaqoob, M.M., et al. Erythropoietin attenuates the tissue injury associated with hemorrhagic shock and myocardial ischemia. Shock. 22 (2004) : 63-69.

[14] Coleman, T., Brines, M., Science review: recombinant human erythropoietin in critical illness: a role beyond anemia? Crit Care. 8 (2004) : 337-341.

[15] Jelkmann, W., Wagner, K., Beneficial and ominous aspects of the pleiotropic action of erythropoietin. Ann Hematol. 83 (2004) : 673-686.

[16] Jelkmann, W. 'O', erythropoietin carbamoylation versus carbamylation. Nephrol Dial Transplant. 23 (2008) : 3033.

[17] Leist, M., Ghezzi, P., Grasso, G., Bianchi, R., Bianchi, R., Villa, P., Fratelli, M., et al. Derivatives of erythropoietin that are tissue protective but not erythropoietic. Science. 305 (2004) : 239-242.

[18] Fiordaliso, F., Chimenti, S., Staszewsky, L., Bai, A., Carlo, E., Cuccovillo, I. et al. A nonerythropoietic derivative of erythropoietin protects the myocardium from ischemia- reperfusion injury. Proc Natl Acad Sci USA. 102 (2005) : 2046-2051.

[19] Moon, C., Krawczyk, M., Paik, D., Coleman, T., Brines, M., Juhaszova, M. et al. Erythropoietin, modified to not stimulate red blood cell production, retains its cardioprotective properties. J Pharmacol Exp Ther. 316 (2006) : 999–1005.

[20] Coleman, T.R., Westenfelder, C., Tögel, F.E., Yang, Y., Hu, Z., Swenson, L. et al. Cytoprotective doses of erythropoietin or carbamylated erythropoietin have markedly pro-coagulant and vasoactive activities. Proc Natl Acad Sci USA. 106 (2006) : 5965–5970.

[21] Mennini, T., De Paola, M., Bigini, P., Mastrotto, C., Fumagalli, E., Barbera, S. et al. Nonhematopoietic erythropoietin derivatives prevent motoneuron degeneration in vitro and in vivo. Mol Med. 12 (2006) : 153–160.

[22] Imamura, R., Isaka, Y., Ichimaru, N., Takahara, S., Okuyama, A. Carbamylated erythropoietin protects the kidney from ischemia-reperfusion injury without stimulating erythropoiesis. Biochem Biophys Res Commun. 353 (2007) : 786-792.

[23] King, V.R., Averill, S.A., Hewazy, D., Priestley, J.V., Torup, L., Michael-Titus, A.T. Erythropoietin and carbamylated erythropoietin are neuroprotective following spinal cord hemisection in the rat. Eur J Neurosci. 26 (2007) : 90-100.

[24] Brines, M., Grasso, G., Fiordaliso, F., Sfacteria, A., Ghezzi, P., Fratelli, M. et al. Erythropoietin mediates tissue protection through an erythropoietin and common β–subunit heteroreceptor. Proc Natl Acad Sci USA. 101 (2004) : 14907-14912.

[25] Satake, R., Kozutsumi, H., Takeuchi, M., Asano, K. Chemical modification of erythropoietin : an increase in in vivo activity by guanidination. Biochim BiophysActa. 1038 (1990) : 125-129.

[26] Jelkmann, W. Recombinant EPO production points the nephrologist should know. Nephrol Dial Transplant. 22 (2007) : 2749 – 2753.

[27] Lin, F.K., Suggs, S., Lin, C.H., Browne, J.K., Smalling, R., Egrie, J. C., et al. Cloning and expression of the human erythropoietin gene. Proc Natl Acad Sci U S A. 82 (1985) : 7580–7584.

[28] U.S. Food and Drug Administration. FDA Drug Safety Communication: Erythropoiesis-Stimulating Agents (ESAs): Procrit, Epogen and Aranesp. Available online at:http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm200297.htm [2010, April 8]

[29] Ehrenreich, H., Hasselblatt, M., Dembowski, C., Cepek, L., Lewczuk, P., Steifel, M., et al. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med. 8 (2002) : 495–505.

[30] Brines, M., Cerami, A. Emerging biological roles for erythropoietin in the nervous system. Nat Rev Neurosci. 6 (2005) : 484-494.

[31] Jelkmann, W. *Erythropoietin after a century of research:younger than ever*. European Haematology. 78 (2007) : 183–205.

[32] Cheetham, J.C., Smith, D.M., Aoki, K.H., Stevenson JL., Hoeffel, T.J., Syed, R.S., et al. TS: NMR structure of human erythropoietin and a comparison with its receptor bound conformation. Nat Struct Biol. 5 (1998) : 861–866.

[33] Masuda, S., Nagao, M., Takahata, K., Konishi, Y., Gallyas, F. Jr., Tabira, T., et al. Functional erythropoietin receptor of the cells with neural characteristics: comparison with receptor properties of erythroid cells. J Biol Chem. 268 (1993) : 11208–11216.

[34] Sadamoto, Y., Igase K, Sakanaka M, Sato, K., Otsuka, H., Sakaki, S., et al. Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery. Biochem Biophys Res Commu. 253 (1998) : 26–32.

[35] Bernaudin, M., Marti, H.H., Roussel, S., Divoux, D., Nouvelot, A., MacKenzie, E.T., et al. A potential role for erythropoietin in focal permanent cerebral ischemia in mice. J Cereb Blood Flow Metab. 19 (1999) : 643–651.

[36] Celik, M., Gokmen, N., Erbayraktar, S., Akhisaroglu, M., Konakc, S., Ulukus, C., et al. Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. Proc Natl Acad Sci U S A. 99 (2002) : 2258–2263.

[37] Olsen, N.V. Central nervous system frontiers for the use of erythropoietin. Clin Infect Dis . 37 (2003) (Suppl 4) : S323–S330.

[38] Ehrenreich, H., Hasselblatt, M., Dembowski, C., Cepek, L., Lewczuk, P., Stiefel M et al. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med. 8 (2002) : 495–505.

[39] Grasso, G., Buemi, M., Alafaci, C., Sfacteria, A., Passalacqua, M., Sturiale, A. et al. Beneficial effects of systemic administration of recombinant human erythropoietin in rabbits subjected to subarachnoid hemorrhage. Proc Natl Acad Sci U S A. 99 (2002) : 5627–5631.

[40] Chong, Z.Z., Kang, J.Q., Maiese, K. Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. Circulation. 106 (2002) : 2973–2979.

[41] Shingo, T., Sorokan, S.T., Shimazaki, T., Weiss S. Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. J Neurosci. 21 (2001) : 9733–9743.

[42] Digicaylioglu, M., Lipton, S.A. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature. 412 (2001) : 641–647.

[43] Yamasaki, M., Mishima, H.K., Yamashita, H., Kashiwagi, K., Murata, K., Minamoto, A. et al. Neuroprotective effects of erythropoietin on glutamate and nitric oxide toxicity in primary cultured retinal ganglion cells. Brain Research. 1050 (2005) : 15 – 26.

[44] Johnson, D.W., Pat, B., Vesey, D.A., Guan, Z., Endre, Z., Gobe, G.C. Delayed administration of darbepoetin or erythropoietin protects against ischemic acute renal injury and failure. Kidney Int. 69 (2006) : 1806-1813.

[45] Vesey, D.A., Cheung, C., Pat, B., Endre Z, Gobé G, Johnson DW. Erythropoietin protects against ischaemic acute renal injury. Nephrol Dial Transplant. 19 (2004) : 348-355.

[46] Sharples EJ, Patel N, Brown P, Stewart, K., Mota-Philipe, H., Sheaff, M. et al. Erythropoietin protects the kidney against the injury and dysfunction caused by ischemia-reperfusion. J Am Soc Nephrol. 15 (2004) : 2115-2124.

[47] Spandou, E., Tsouchnikas, I., Karkavelas, G., Dounousi, E., Simeonidou, C., Guiba-Tziampiri, O. et al. Erythropoietin attenuates renal injury in experimental acute renal failure ischaemic/reperfusion model. Nephrol Dial Transplant. 21 (2006) : 330-336.

[48] Johnson, D.W., Pat, B., Vesey, D.A., Guan, Z., Endre, Z., Gobe, G.C. Delayed administration of darbepoetin or erythropoietin protects against ischemic acute renal injury and failure. Kidney Int. 69 (2006) : 1806-1813.

[49] Bahlmann, F.H., Song, R., Boehm, S.M., Mengel, M., von Waseljewski, R., Lindschau, C. et al. Low-dose therapy with the long-acting erythropoietin analogue darbepoetin alpha persistently activates endothelial Akt and attenuates progressive organ failure. Circulation. 110 (2004) : 1006-1012.

[50] Kang, D.H., Park, E.Y., Yu, E.S., Lee, Y.S., Yoon, K.I. Renoprotective effect of erythropoietin (EPO): possibly via an amelioration of renal hypoxia with stimulation of angiogenesis in the kidney. Kidney Int. 67 (2005) : 1683-1690.

[51] Vaziri, N.D., Zhou, X.J., Liao, S.Y. Erythropoietin enhances recovery from cisplatin-induced acute renal failure. Am J Physiol. 266 (1994) : F360-6.

[52] Bagnis, C., Beaufils, H., Jacquiaud, C., Adabra, Y., Jouanneau, C., Le Nahour, G., et al. Erythropoietin enhances recovery after cisplatin-induced acute renal failure in the rat. Nephrol Dial Transplant. 16 (2001) : 932-939.

[53] Goldfarb, M., Rosenberger, C., Ahuva, S., Rosen, S., Heyman, H.N. A role for erythropoietin in the attenuation of radiocontrast-induced acute renal failure in rats. Ren Fail. 28 (2006) : 345-350.

[54] Chang, Y.K., Choi, D.E., Na, K.R., Lee, S.J., Suh, K.S., Kim, S.Y., et al. Erythropoietin Attenuates Renal Injury in an Experimental Model of Rat Unilateral Ureteral Obstruction via Anti-Inflammatory and Anti-Apoptotic Effects. Urology. 181 (2009) : 1434-1443.

[55] Toba, H., Sawai, N., Morishita, M., Murata, S., Yoshida, M., Nakashima, K., et al. Chronic treatment with recombinant human erythropoietin exerts renoprotective effects beyond hematopoiesis in streptozotocin- induced diabetic rat. Eu J of Phamacology. 612 (2009) : 106-114.

[56] Cai, Z., Manalo, D.J., Wei, G., Rodriguez, E.R., Fox-Talbot, K., Lu, H., et al. Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. Circulation. 108 (2003) : 79–85.

[57] Joyeux-Faure, M., Ramond, A., Beguin, P.C., Belaidi, E., Godin-Ribuot, D., Ribuot, C. Early pharmacological preconditioning by erythropoietin mediated by inducible NOS and mitochondrial ATP-dependent potassium channels in the rat heart. Fundam ClinPharmacol. 20 (2006) : 51–56.

[58] Parsa, C.J., Matsumoto, A., Kim, J., Riel, R.U., Pascal, L.S., Walton, G.B., et al. A novel protective effect of erythropoietin in the infarcted heart. J Clin Invest. 112 (2002) : 999–1007.

[59] Lipsic, E., vander meer, P., Henning, R.H, Suurmeijer, A.J., Boddeus, K.M., van Veldhuisen, D.J., et al. Timing of erythropoietin treatment for cardioprotection in ischemia/reperfusion. J Cardiovasc Pharmacol. 44 (2004) : 473– 479.

[60] Chong, Z.Z., Kang, J.Q., Maiese, K. Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation ofcysteine proteases. Circulation. 106 (2002) : 297-2979.

[61] Burger, D., Lei, M., Geoghegan-Morphet, N., Lu, X., Xenocostas, A., Feng, Q. Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase. Cardiovasc Res. 72 (2006) : 51-59.

[62] Hamed, S., Barshack, I., Luboshits, G., Wexler, D., Deutsch, V., Keren, G., et al. Erythropoietin improves myocardial performance in doxorubicin-induced Cardiomyopathy. Eur Heart J. 27 (2006) : 1876- 1883.

[63] Li, L., Takemura, G., Li, Y., Miyata, S., Esaki, M., Okada, H., et al. Preventive effect of erythropoietin on cardiac dysfunction in doxorubicin-induced cardiomyopathy. Circulation. 113 (2006) : 535-543.

[64] Westenbrink, B.D., Oeseburg, H., Kleijn, L., van der Harst, P., Belonje, A.M., Voors,A.A., et al. Erythropoietin stimulates normal endothelial progenitor cell-mediated endothelial turnover, but attributes to neovascularization only in the presence of local ischemia. Cardiovasc Drugs Ther. 22 (2008) : 265-274.

[65] Lipsic, E., Westenbrink, B.D., van der Meer, P., van der Harst, P., Voors, A.A., van Veldhuisen, D.J.,et al. Low-dose erythropoietin improves cardiac function in experimental heart failure without increasing haematocrit. Eur J Heart Fail. 10 (2008) : 22-9.

[66] Yilmaz, S., Ates, E., Tokyol, C., Pehlivan, T., Erkasap, S., Koken, T.The protective effect of erythropoietin on ischaemia/reperfusion injury of liver. Healthscience. 6 (2004) : 169-173.

[67] Sepodes, B., Maio, R., Pinto, R., Sharples, E., Oliveira, P., McDonald, M., et al. Recombinant human erythropoietin protects the liver from hepatic Ischemia reperfusion injury in the rats. Transpl Int, 19 (2006) : 919–926.

[68] Yu, H.L., Zheng, D.L., Li, X.L.. Dong, G.H. Pretreatment with erythropoietin reduces hepatic ischemia-reperfusion injury. Hepatobiliary Pancreat Dis Int. 8 (2009) : 294-299.

[69] Schmeding, M., Neuman, U.P., Baas-Knoop, S., Spinelli, A., Neuhaus, P. Erythropoetin reduces ischemia-reperfusion injuring in the rat liver. Eur Surg Res. 39 (2007) : 189–197.

[70] Madro, A., Kurzepa, J., Czechowska, G., et al. Erythropoietin inhibits liver gelatinases during galactosamine-induced hepatic damage in rats. Phamacological Report. 61 (2009) : 917-923.

[71] Sigal M, Siebert N, Zechner D, Slomka, M., Celinski, K., Szymonik-Lesiuk, S. Darbepoetin-a inhibits the perpetuation of necro-inflammation and delays the progression of cholestatic fibrosis in mice. Laboratory Investigation. 90 (2010) : 1447–1456.

[72] Mun, K.C., Golper, T.A. Impaired biological activity of erythropoietin by cyanate Carbamylation. Blood Purif. 18 (2000):13–17.

[73] Carla, J.S.M., Fernanda, S., Georg, G., Artur, C-P. Chemical modifications on proteins using glutaraldehyde. Food Technol Biotechnol. 42 (2004) : 51-56.

[74] Kawaguchi, Y., Ito, A., Appella, E., Yao, T. Charge modification at multiple C-terminal lysine residues regulates p53 oligomerization and its nucleus-cytoplasm trafficking. J Biol Chem. 281 (2006) : 1394–1400.

[75] Horkko, S., Savolainen, M.J., Kervinen, K., , Kesaniemi, Y.A . Carbamylation-induced alterations in low-density lipoprotein mechanism. Kidney Int. 4 (1992) : 1175- 1181.

[76] Park, K.D., Mun, K.C., Chang, E.J., Park, S.B., Kim, H.C. Inhibition of erythropoietin activity by cyanate. Scand J Urol Nephrol. 38 (20 04): 69–72.

[77] Michèle, R.R., Charis, G. N-Acetylbenzotriazole as a Protein Reagent. Eur J Biochem. 65 (1976) : 25–33.

[78] Habeeb, A.F., Cassidy, H.G., Singer, S.G. Molecular structural effects produced in proteins by reaction with succinic anhydride. Biochem Biophys Acta. 29 (1958) : 587-593.

[79] Carla, J.S.M., Fernanda, S., Georg, G., Artur, C-P. Chemical modifications on proteins using glutaraldehyde. Food Technol Biotechnol. 42 (2004) : 51-56.

[80] Cupo, P., EI-Deiry, W., Whitney, P.L., Awad, W.M. Stabilization of protein by guanidination. J Biol Chem. 255 (1980) : 10828–10833.

[81] Lundblad, R.L., Noyes, C.M. The modification of lysine. In: Lundblad RL and Noyes CM (ed). CRC Press: Florida. 1984

[82] Fields R. The measurement of amino groups in proteins and peptides. Biochem J. 1971 (124): 581-590.

[83] Sirén, A.L., Fratelli, M., Brines, M., Goemans, C., Casagrande, S., Lewczuk, P.,et al. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci U S A. 98 (2001) : 4044- 4049.

[84] Manotham, K., Tanaka, T., Ohse, T., Kojima, I., Miyata, T., Inagi, R. et al. A biologic role of HIF-1 in the renal medulla. Kidney Int. 67 (2005) : 1428-1439.

[85] Combe, C., Tredree, R.L., Schellekens, H. Biosimilar, epoetins: an analysis based on recently implemented European medicines evaluation agency guidelines on comparability of biopharmaceutical proteins. Pharmacotherapy. 25 (2005) : 954-962.

[86] Villalobos, A.P., Gunturi, S.R., Heavner, G.A. Interaction of polysorbate 80 with erythropoietin: a case study in protein–surfactant interactions. Pharm Res. 22 (2005) : 1186-1194.

[87] Dumont, A. Hydrogen peroxide-induced apoptosis is CD 95-independent, requires the release of mitochondria-derived reactive oxygen species and the activation of NF-kappa B. Oncogene. 18 (1999) : 747-757.

[88] Kim, H., Lee, T.H., Park, E.S., Suh, J.M., Park, S.J., Chung, H.K., et al. Role of peroxiredoxins in regulating intracellular hydrogen peroxide and hydrogen peroxide-induced apoptosis in thyroid cells. J Biol Chem. 275 (2000) : 18266-18270.

[89] Liu, C.L., Xie, .LX., Li, M., Durairajan, S.K., Goto, S., Huan, J.D.. Salvianolic Acid B Inhibits Hydrogen Peroxide-Induced Endothelial Cell Apoptosis through Regulating PI3K/Akt Signaling. PLoS One. 19 (2007) : e1321.

[90] Galli, G., Fratelli, M. Activation of apoptosis by serum deprivation in a teratocarcinoma cell line: inhibition by L-acetylcarnitine. Exp Cell Res . 1204 (1993) : 54-60.

[91] Ninomiya, Y., Adams, R., Morriss-Kay, G.M., Eto, K. Apoptotic cell death in neuronal differentiation of P19 EC cells: cell death follows reentry into S phase. J Cell Physiol. 172 (1997) : 25-35.

[92] Patel, N.S., Sharples, E.J., Cuzzocrea, S., Chatterjee, P.K., Britti, D., Yaqoob, M.M. et al. Pretreatment with EPO reduces the injury and dysfunction caused by ischemia/reperfusion in the mouse kidney in vivo. Kidney Int. 266 (2004) : 983–989.

[93] Gong, H., Wang, W., Kwon, T.H., Jonassen, T., Li, C., Ring, T. et al. EPO and alpha-MSH prevent ischemia/reperfusion-induced down-regulation of AQPs and sodium transporters in rat kidney. Kidney Int. 66 (2004) : 683–695.

[94] Menne, J., Park, J.K., Shushakova, N., Mengel, M., Meier, M., Fliser, D. et al. The continuous erythropoietin receptor activator affects different pathways of diabetic renal injury. J Am Soc Nephrol. 18 (2007) : 2046-2053.

APPENDIX

APPENDIX

BUFFERS AND REAGENTS

1. 2 M cyanate solution

Sodium cyanate	130.02	g.
Distilled water	1000	ml.

2. 100 mM phosphate buffer pH 7

2M KH_2PO_4	21.1	ml.
2M K_2HPO_4	28.9	ml.

Adjust volume to 1000 ml with distilled water.

3. 0.5 M NaHCO3 (pH 8.0)

NaHCO3	42	g.
Distilled water	1000	ml.

4. 10 mM sodium acetate

sodium acetate	1.36	g.
Distilled water	1000	ml.

5. 5 mM calcium acetate buffer pH 7.5

calcium acetate	0.088	g.

Dissolve in distilled water and adjust pH to 7.5 with glacial acetic acid.

Distilled water	100	ml.

6. 1M Tris- HCl pH 8.4

Tris	12.11	g.

Dissolve in distilled water and adjust pH to 8.4 with HCl.

Distilled water to	100	ml.

7. 1 M O-methyl isourea

O-methyl isourea	12.31	g.
Distilled water	100	ml.

8. RIPA buffer

NaCl	0.876	g.
Triton-x	1	ml.
Sodium deoxycholate	0.5	g.
Sodium dodecyl sulfate	0.1	g.
Tris	6.05	g.
Distilled water to	100	ml.

BIOGRAPHY

Miss Supreecha Chattong was born on June 27, 1979 in Singburi Province. She graduated Master Degree Program of Science, major biotechnology from Chulalongkorn University in 2006. She now has a position as scientist at Lerdsin General Hospital.