【S-13】

Toxicological Assessment using Non-Human Primate for Biotherapeutics

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The non-human primates are considered to be the most suitable model animals for the safety /efficacy evaluation of the newly developed biotherapeutics, because they share many features of biology and structure with humans.

In my talk, the key considerations for safety assessment using non-human primate will be presented as follows; 1) why we need non-human primate for newly developed biotherapeutics, 2) what we prepare in advance for a monkey study (husbandry, management, experimental methodology, index of acclimatization such as transport stress and heavy metal level in blood, 3) what we consider for safety assessment (physiological levels in hematology, serum biochemistry, relative organ weight, circadian rhythm of locomotion, and effect of ketamine anesthesia on biological variation in blood, 4) example toxicity study in cynomolgus monkeys using EPO.

Toxicological Assessment using Non-human Primate for Biotherapeutics

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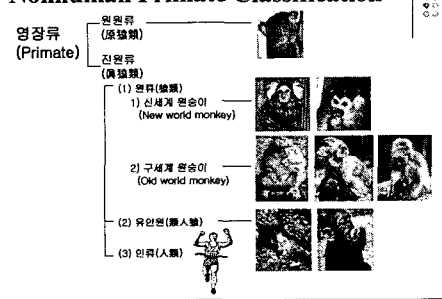
What is key considerations for safety assessment using non-human primate ?

1. Why we need non-human primate for newly developed biotherapeutics ?
2. What we prepare in advance for a monkey study ?
   - Husbandry, Management, Experimental methodology
   - Index of acclimatization for newly acquired monkey
3. What we consider for safety assessment ?
   - Physiological levels in hematology, serum biochemistry, relative organ weight, and spontaneously histopathological lesions
   - Clinical signs (e.g., locomotion, circadian rhythm of locomotion)
   - Effect of ketamine anesthesia on biological variation of blood parameter
4. Example toxicity study in cynomolgus monkeys using EPO

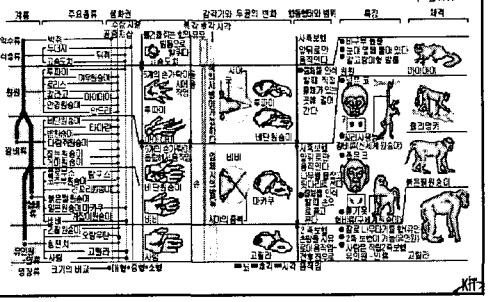
Nonhuman primate
: Closest animal relatives to humans

- similar biological systems
- probability of similar reaction is very high

Nonhuman Primate Classification



Summary of Characteristics



Selection of Animal Model

1. Marmoset
2. Cynomolgus Monkey
   Young adult : Safety Assessment
   Mature : Reproduction physiology
   Aged monkey : Osteoporosis research

Test Substances in Safety Assessment using Non-human Primate

- Antibiotic
- Anti-malarial
- **Biotech products (EPO, Interferons, rhGH, anti AIDS)**
- Calcium antagonist
- Cephalosporin
- Dopamine antagonist
- Estrogen
- Fungicide
- Herbicide
- Progstaglandin
- NSAID
- Steroid anti-hormone
- Sulphonamide

2. What we prepare in advance for a monkey study ?

- Husbandry
  - General consideration for primate housing facility
  - Cage design consideration
- Management
  - AAALAC, IACUC
  - Zoonoses
- Experimental methodology
  - Restraint, blood sampling technique, administration technique
- Index of acclimatization for newly acquired monkey
  - Transport stress
  - Heavy metal level in blood

Indirect Indicator of Transport Stress in Hematological Values in Newly Acquired Cynomolgus Monkeys

☐ **Study history**

- It is well known that plasma cortisol level increased in proportion to the extent of stress.

- The increased cortisol level caused an increase in neutrophil to lymphocyte (N/L) ratio by destructing lymphocytes in the thymus cortex or extending neutrophil half-life.

- A previous study of transport stress in rabbit showed an increase in N/L ratio and elevated level of cortisol immediately on arrival day.

(Kim et al. 2005 : J. Med. Primatol. 34:188-192)

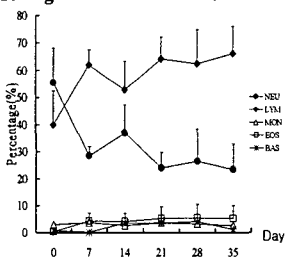
☐ Materials & Methods

1) Animals
- Strain: cynomolgus monkey
- No. animal, Sex and age : Five male monkey (3- 4 years)

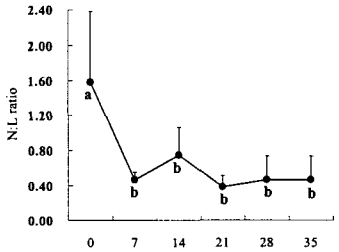
2) Transport Condition
- They underwent air and ground travel-related stress in transport cages for a 15 hour- transit time in the transport cage
: Cage dimension : 310W x 335L x 585H mm

3) Blood Sampling
- Blood samples were obtained from the cephalic vein within 30 minutes of an intramuscular injection of ketamine hydrochloride (10 mg/kg).

Changes in Differential Count of WBC

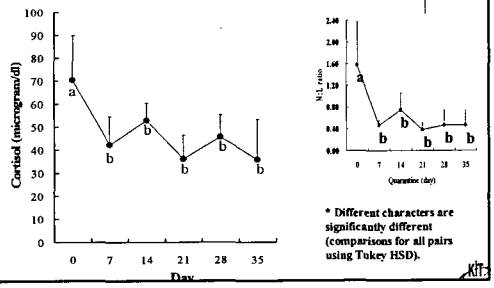


Change in neutrophil to lymphocyte (N/L) ratio



* Different characters are significantly different (comparisons for all pairs using Tukey HSD).

Serum cortisol levels vs. N/L ratio



* Different characters are significantly different (comparisons for all pairs using Tukey HSD).

Heavy metals in blood

□ Object
: to ascertain the physiological level of heavy metal (Cd, Pb) in newly cynomolgus monkey

1) Animals : Cynomolgus monkeys (n=124)
- Age : 3 ~ 5 years, Yunnan National Primate Research Center (China)
2) Analysis items : Cd and Pb in Arrival vs. 1 month after arrival
3) Analysis conditions
- Analysis instrument : AAS (AAnalyst 600, Perkin-Elmer, USA)

	Pb	Cd
Sample	Whole blood	Whole blood
Sample treatment Matrix modifier	0.2% Triton X-100 0.2% $(NH_4)_2HPO_4$	0.2% Triton X-100 0.2% $(NH_4)_2HPO_4$
Dilution	*1:20*	*1:20*
Dring temp	90 ~ 130℃	90 ~ 130℃
Ashing temp	300 ~ 500℃	300 ~ 450℃
Atomizing temp	1600℃	1500℃
Wavelength	283.3 nm	228.8 nm

Levels of heavy metals in blood

	Arrival	1 month after arrival	Human
Pb (μg/dl)	2.421 ± 2.497*	1.107 ± 1.023***	5 ~ 12 (Male) 3 ~ 7 (Female)
Cd (μg/dl)	0.045 ± 0.064	0.033 ± 0.023*	0.02 ~ 0.10 (Non-smoker) 0.11 ~ 0.36 (Smoker)

* Mean ± SD; n= 124 (male and female), *p < 0.05 , ** p <0.01, *** p <0.001

Summary

1. It is possible that an increase in N/L ratio may be utilized as an indirect indicator of transport stress in newly acquired cynomolgus monkeys, since it has the similar pattern of change in cortisol.

2. It takes at least 1 month for acclimatization, since heavy metals (e.g., Cd, Pb) decreased at 1 month after arrival.

3. What we consider for safety assessment ?

- Physiological levels in hematology, serum biochemistry, relative organ weight, and spontaneously histopathological lesions
- Clinical signs
  - *locomotion, circadian rhythm of locomotion*
- Effect of ketamine anesthesia on biological variation in blood parameter

Physiological levels in hematological parameters

□ Object
: to ascertain the physiological level of hematological and serum biochemical parameter in newly cynomolgus monkey.
1. Arrival : 2004. 12. 14
2. Animals : 134 monkeys (Male 72, Female 64)
3. Source : Yunnan National Primate Research Center (China)

	Male	Female	Human range
Red blood cell count (10^6/μl)	5.44 ± 0.38*	5.17 ± 0.40	4.2 ~5.5
Hemoglobin (g/dl)	12.70 ± 0.89	12.10 ± 0.85	13 ~17
Hematocrit (%)	41.9 ± 2.84	40.5 ± 2.46	39~54
MCV (fl)	77.2 ± 3.38	78.5 ± 3.02	84 ~98
MCH (pg)	23.3 ± 0.98	23.5 ± 0.88	32 ~36
MCHC (g/dl)	30.2 ± 0.91	29.9 ± 0.84	28 ~34
Platelet count (10^3/μl)	401.0 ± 89.4	399.0 ± 112.8	160 ~400
RET (%/100 red blood cells)	1.90 ± 0.67	1.80 ± 0.59	-
White blood cell count (10^3/μl)	9.78 ±3.30	10.04 ± 3.53	4.0 ~10.8

Physiological level in serum biochemical parameters

	Male	Female	Human range
AST (IU/l)	35.60 ± 7.85	40.90 ± 46.33	0 ~ 37
ALT (IU/l)	38.80 ± 16.07	49.2 ± 46.33	0 ~ 40
ALP (IU/l)	949.0 ± 332.9	748.0 ± 235.3	84 ~ 279
BUN (mg/dl)	17.30 ± 3.56	18.10 ± 3.57	7 ~ 21
Creatinine (mg/dl)	0.82 ± 0.16	0.77 ± 0.11	0.6 ~ 1.2
Glucose (mg/dl)	72.6 ± 15.94	64.7 ± 11.71	70 ~ 110
T-cholesterol (mg/dl)	117.5 ± 25.99	114.0 ± 26.47	150 ~ 230
Albumin/globulin (ratio)	1.49 ± 0.22	1.47 ± 0.17	0.9 ~ 2.2
Total protein (g/dl)	6.94 ± 0.43	7.08 ± 0.42	6.6 ~ 8.2
Albumin (g/dl)	4.12 ± 0.22	4.20 ± 0.23	3.9 ~ 5.1
CPK (IU/l)	649 ± 504	868 ± 857	49 ~ 257
Triglyceride (mg/dl)	19.8 ± 7.8	23.8 ± 8.8	10 ~ 200
Total bilirubin (mg/dl)	0.18 ± 0.05	0.22 ± 0.05	0.3 ~ 1.5
Inorganic phosphate (mg/dl)	6.06 ± 0.76	6.17 ±0.90	2.5 ~ 5.0
Calcium (mg/dl)	9.17 ± 0.45	9.07 ± 0.37	-
Chloride (mmol/l)	109.0 ± 2.9	109.0 ± 2.9	97 ~ 110
Sodium(mmol/l)	147.0 ± 1.7	147.0 ± 1.7	135 ~ 145
Potassium (mmol/l)	4.06± 0.29	4.07 ± 0.37	-

* Mean ± SD; n= 71 (male) or 63 (female)

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Physiological levels in relative organ weights

☐ Object
: to ascertain the physiological level of relative organ weight in control monkeys.

Organs (% Body weight)	Male	Female
Body weight	3908 ± 913 (n=11)	3105 ± 374 (n=6)
Salivary gland	0.069 ± 0.013	0.064 ± 0.020
Spleen	0.106 ± 0.039	0.103 ± 0.028
Thyroid gland	0.011 ± 0.004	0.011 ± 0.005
Testis	0.173 ± 0.162	-
Seminal vesicle	0.046 ± 0.037	-
Prostate	0.013 ± 0.009	-
Ovarys	-	0.009 ± 0.004
Uterus		0.098 ± 0.028
Liver	1.569 ± 0.309	1.892 ± 0.238
Lung	0.470 ± 0.056	0.474 ± 0.039
Brain	1.797 ± 0.431	1.830 ± 0.322
Pituitary gland	0.001 ± 0.000	0.002 ± 0.001
Thymus	0.079 ± 0.054	0.085 ± 0.028
Heart	0.362 ± 0.034	0.361 ± 0.037
Kidneys	0.322 ± 0.031	0.403 ± 0.047
Adrenal gland	0.012 ± 0.004	0.014 ± 0.002

* Each value represents as mean ± SD.

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Comparisons in relative organ weights

Species	BW(kg)	Brain(%)	Heart(%)	Liver(%)	Kidney(%)	Testis(%)
사람	M: 42-84 F: 46	1.76-3.02	0.42-0.81	2.30-2.81	0.37-0.51	0.04
Cynomolgus monkey*	M: 3.9 F: 3.1	1.80±0.43 *1.83±0.32*	0.36±0.03 *0.36±0.04*	1.57±0.31 *1.89±0.24*	0.32±0.03 *0.40±0.05*	0.17±0.16
개	M: 13.2-18.9 F: 12.4-16.5	0.42-0.65	0.55-0.78	1.95-3.30	0.31-0.54	0.15-0.28
토끼	M: 2.8 F: 2.5	0.36-0.37	0.2	2.87-3.27	0.51-0.52	0.11
랫드	M,F: 0.25	1.22	0.52	3.35	1.09	0.87

* Mean ± SD, male (n=11), female (n=6)

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Circadian rhythm in Locomotion

☐ Object
: to ascertain the physiological level of locomotor activity in cage

1. Animal
- Cynomolgus monkey : male 9, female 9
- Body weight : 3.77 ± 0.42 (Male), 3.18 ± 0.31 (Female)
- Age : 3.4 ~ 4.7 year (Male), 3.3 ~ 5.2 year (Female)

2. Cage Locomotion
- Software: Vigie Primate
- Cage size : 450W x 650L x 754H mm
- Lighting condition : 07:00 – 19:00 (Light)

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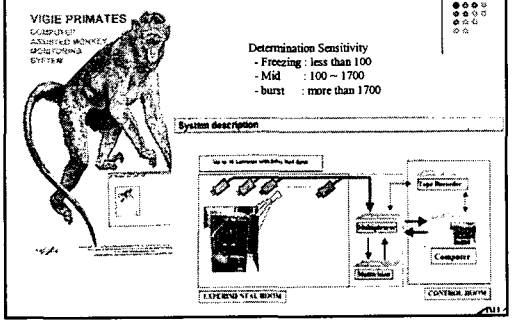

Locomotor Activity in Light and Dark Period

(locomotion unit: count)

	Male		Female	
	Dark period	Light period	Dark period	Light period
Average	1240 ± 973	12323 ± 9277	3418 ± 2046	16881 ± 9403
D/L ratio	9.14%	90.86%	16.83%	83.16%

* Mean ± SD (male n= 9; female n=9)

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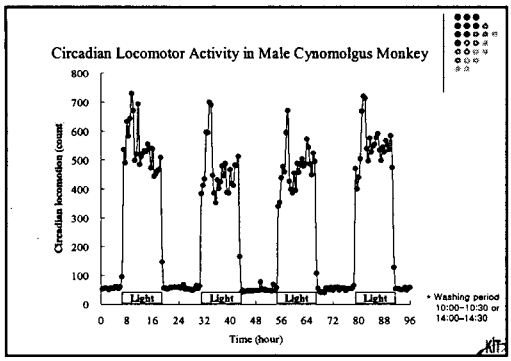

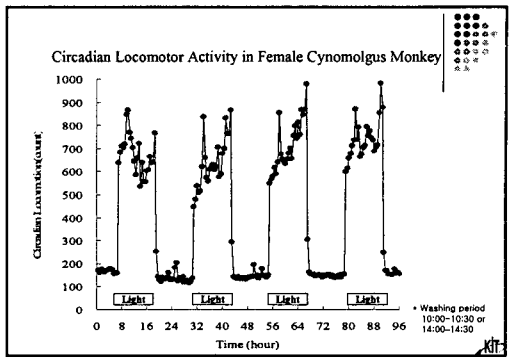

Circadian Locomotor Activity in Cynomolgus Monkeys

(locomotion unit: count)

	Male		Female	
	Dark period	Light period	Dark period	Light period
Day 1	1335 ± 933*	13166 ± 9355	3543 ± 1920	16447 ± 9347
Day 2	1186 ± 787	11378 ± 7884	3162 ± 2007	15603 ± 8082
Day 3	1183 ± 936	11474 ± 9190	3466 ± 2148	17436 ± 9713
Day 4	1258 ± 1234	13276 ± 10678	3500 ± 2110	18038 ± 10471
Average	1240 ± 973	12323 ± 9277	3418 ± 2046	16881 ± 9403
Total activity (D + L)	13563 ± 10250 (100%)		20299 ± 11449 (150%)	

* Mean ± SD (male n= 9; female n=9)

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Effect of Anesthesia by Ketamine on Hematological and Serum Biochemical Values

☐ Study history

- Ketamine hydrochloride is the most commonly used drug for the chemical restraint of nonhuman primates in order to facilitate safe handling especially during quarantine period.

- Although cynomolgus monkeys anesthetized with ketamine hydrochloride are widely utilized for biomedical research, the effects of ketamine anesthesia on hematological and serum biochemical variables have not yet been clarified.

(Kim et al. 2005: J. Med. Primatol. 34:96-100)

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☐ Materials & Methods

1) Animals
- Species : cynomolgus monkey
- Sex and age : male 19, female 16 (3- 5 years)

2) Anesthesia
- Ketamine Hydrochloride : 10 mg/kg (IM)

3) Blood Sampling
- Anesthetic or Non-anesthetic condition
: Blood samples were obtained from the cephalic vein within 30 minutes of an intramuscular injection of ketamine hydrochloride (10 mg/kg).

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Hematological Changes in Male Monkeys

	Non-anesthetic condition*	Anesthetic condition*	% Change
Red blood cell count (10^6/μl)	5.26 ± 0.34	5.56 ± 0.34	+5.7 ***
Hemoglobin (g/dl)	12.65 ± 0.76	13.57 ± 0.75	+7.3 ***
Hematocrit (%)	47.46 ± 2.58	47.02 ± 2.43	-0.9
MCV (fl)	90.30 ± 3.20	84.65 ± 2.76	-6.3 ***
MCH (pg)	24.04 ± 0.66	24.45 ± 0.84	+1.7 **
MCHC (g/dl)	26.64 ± 0.84	28.9 ± 0.94	+8.5 ***
Platelet count (10^3/μl)	420.84 ± 82.11	430.53 ± 92.14	+2.3
RET (%/100 red blood cells)	1.73 ± 0.43	2.02 ± 0.39	+16.8
White blood cell count (10^3/μl)	13.05 ±3.28	9.16 ± 2.69	-29.8 ***
Neutrophils (%)	26.00 ± 10.04	36.12 ± 11.02	+38.9 **
Lymphocytes (%)	67.31 ± 9.47	59.00 ± 10.56	-12.3 **
Monocytes (%)	3.48 ± 0.85	3.03 ± 1.00	-12.9
Eosinophils (%)	0.88 ± 0.43	0.69 ±0.62	-21.6
Basophils (%)	4.27 ± 3.15	0.82 ± 0.97	-80.8 ***

* Mean ± SD: n= 19 (male) or 16 (female). * p < 0.05 , ** p <0.01, *** p <0.001

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Hematological Changes in Female Monkeys

	Non-anesthetic condition *	Anesthetic condition *	% Change
Red blood cell count (10^6/μl)	5.13 ± 0.31	5.35 ± 0.39	+4.3 **
Hemoglobin (g/dl)	12.24 ± 0.79	12.86 ± 1.07	+5.1 **
Hematocrit (%)	46.99 ± 2.42	45.41 ± 3.91	-3.4 *
MCV (fl)	91.67 ± 3.25	84.81 ± 3.67	-7.5 ***
MCH (pg)	23.86 ± 1.15	24.00 ± 0.97	+0.6
MCHC (g/dl)	26.02 ± 0.77	28.32 ± 0.67	+8.8 ***
Platelet count (10^3/μl)	444.63 ± 70.62	462.06 ± 60.24	+3.9
RET (%/100 red blood cells)	1.60 ± 0.52	1.69 ± 0.38	+5.6
White blood cell count (10^3/μl)	11.06 ± 2.20	8.54 ± 3.61	-22.8 **
Neutrophils (%)	28.84 ± 13.78	38.40 ± 12.95	+33.1 **
Lymphocytes (%)	63.3 ± 13.42	55.03 ± 12.66	-13.1 **
Monocytes (%)	3.78 ± 1.35	3.33 ± 1.00	-11.9
Eosinophils (%)	1.63 ± 0.81	2.11 ±1.41	+29.4
Basophils (%)	2.91 ± 1.58	0.41 ± 0.24	-85.9 ***

* Mean ± SD; n= 19 (male) or 16 (female), * p < 0.05 , ** p <0.01, *** p <0.001

Serum Biochemical Changes in Male Monkeys

	Non-anesthetic condition *	Anesthetic condition *	% Change
AST (IU/l)	41.03 ±7.11	64.86 ± 21.29	+58.1 ***
ALT (IU/l)	40.16 ± 20.13	52.17 ± 11.70	+29.9 **
ALP (IU/l)	1923.0 ± 371.1	1829.2 ± 389.5	-4.9
BUN (mg/dl)	17.67 ± 2.85	18.33 ± 4.11	-3.7
Creatinine (mg/dl)	0.85 ± 0.10	0.79 ± 0.13	-7.1
Glucose (mg/dl)	83.63 ± 12.98	63.68 ± 19.61	-23.9 ***
T-cholesterol (mg/dl)	126.13 ± 21.40	196.86 ± 64.29	+56.1 ***
Albumin/globulin (ratio)	1.58 ± 0.15	1.50 ± 0.11	-5.1 **
Total protein (g/dl)	7.53 ± 0.46	7.43 ± 0.63	+1.3
Albumin (g/dl)	4.60 ± 0.246	4.45 ± 0.34	-3.3
CPK (IU/l)	194.68 ± 53.59	732.16 ± 362.58	+276.1 ***
Triglyceride (mg/dl)	25.18 ± 7.20	14.36 ± 10.16	-43 **
Total bilirubin (mg/dl)	0.18 ± 0.03	0.29 ± 0.07	+61.0 ***
Inorganic phosphate (mg/dl)	7.02 ± 0.90	6.03 ±0.89	-14.1 **
Calcium (mg/dl)	2.91 ± 1.58	0.41 ± 0.24	-85.9 ***
Chloride (mmol/l)	106.68 ± 2.06	108.84 ± 1.80	+2.0 **
Sodium(mmol/l)	152.47 ± 3.08	147.47 ± 2.87	-3.3 ***
Potassium (mmol/l)	5.20 ± 0.46	4.68 ± 0.53	-10 **

Serum Biochemical Changes in Female Monkeys

	Non-anesthetic condition *	Anesthetic condition *	% Change
AST (IU/l)	36.8 ±8.72	50.83 ± 20.89	+38.1 *
ALT (IU/l)	42.49 ± 11.23	72.48 ± 51.03	+70.6 *
ALP (IU/l)	524.19 ± 188.01	479.0 ± 176.9	-8.6 **
BUN (mg/dl)	15.16 ± 1.69	20.06 ± 4.77	+32.3
Creatinine (mg/dl)	0.84 ± 0.12	0.84 ± 0.11	0
Glucose (mg/dl)	87.41 ± 18.16	73.61 ± 24.53	-15.8 *
Total cholesterol (mg/dl)	129.71 ± 27.37	137.64 ± 33.15	+6.1
Albumin/globulin (ratio)	1.35 ± 0.12	1.32 ± 0.14	-2.2
Total protein (g/dl)	7.86 ± 0.43	7.73 ± 0.47	-1.7
Albumin (g/dl)	4.51 ± 0.22	4.39 ± 0.22	-2.7
Creatine phosphokinase(IU/l)	126.31± 44.45	236.50 ± 136.57	+87.2 **
Triglyceride (mg/dl)	32.58 ± 10.36	34.71 ± 53.02	+6.5
Total bilirubin (mg/dl)	0.17 ± 0.03	0.20 ± 0.08	+17.6
Inorganic phosphate (mg/dl)	5.85 ± 0.92	4.11 ±1.20	-29.7 ***
Calcium (mg/dl)	9.51 ± 0.52	9.35 ± 0.50	-1.7
Chloride (mmol/l)	107.63 ± 2.39	105.75 ± 2.14	-1.7 *
Sodium(mmol/l)	152.69 ± 3.68	147.75 ± 2.27	-3.2 ***
Potassium (mmol/l)	5.30 ± 0.71	4.81 ± 0.66	-9.2 **

Summary

1. Ascertaining the physiological level in hematological and serum biochemical parameter in newly cynomolgus monkey and relative organ weight in control monkeys.

2. Considering the physiological level of locomotor activity in cage
   - Locomotor activity in cage shows a cyclicity of circadian rhythmn
   - Locomotor activity in cage is more greater in males in light period, while it is more greater in females in dark period.

3. Effect of ketamine anesthesia should be considered when designing studies for and interpreting data from cynomolgus monkeys
   (decreases in WBC and glucose, P, Na, K; increases in AST, ALT, and CPK)

4. Example toxicity study in cynomolgus monkeys using EPO

Effects of Recombinant Human Erythropoietin Treatment in Male Cynomolgus Monkeys

1. Animal
   - Six cynomolgus monkeys (*Macaca fascicularis*),
   - Grouping : treatment (3 males) and control groups (3 males)
   - Age & Body weight : 4 ~ 5 years, average 4796 g
2. Test item
   : Recombinant human erythropoietin (rHuEPO)
3. Administration & Dose : Intravenously administered 3 times per week at doses of 0 and 2730 IU/ 0.1 ml /kg with rHuEPO for 4 weeks.
4. Determination
   : body weight, organ weight, hematology, serum biochemistry, and histopathology

Changes in relative organ weights in cynomolgus monkeys

Organs (% Body weight)	Control	EPO
Salivary gl.	0.082 ± 0.007	0.161 ± 0.004***
Spleen	0.097 ± 0.038	0.171 ± 0.009*
Thyroid gl.	0.009 ± 0.004	0.007 ± 0.004
Testis	0.311 ± 0.153	0.421 ± 0.171
Epididymis	0.066 ± 0.025	0.074 ± 0.014
Liver	1.313 ± 0.140	1.701 ± 0.012*
Lung	0.441 ± 0.056	0.479 ± 0.050
Brain	1.409 ± 0.255	1.727 ± 0.166
Pituitary gl.	0.001 ± 0.000	0.001 ± 0.000
Thymus	0.053 ± 0.032	0.074 ± 0.025
Heart	0.371 ± 0.037	0.388 ± 0.026
Kidneys	0.304 ± 0.008	0.379 ± 0.021*
Adrenal gl.	0.010 ± 0.003	0.011 ± 0.004

Each value represents as mean ± SD (n= 3).
Significant difference from each VC group (* p < 0.05, ** p < 0.01, *** p < 0.001).

Changes in WBC-related parameters in cynomolgus monkeys

	Days	WBC count (x1000/μl)	LYM(%)	NEU(%)	EOS(%)	BAS(%)	MON(%)
Control	D 0	13.89 ± 1.91	49.1 ± 19.5	44.8 ± 19.5	1.0 ± 0.3	1.0 ± 0.3	2.7 ± 0.4
	D 3	12.54 ± 1.78	42.0 ± 17.0	53.8 ± 17.9	0.5 ± 0.3	1.1 ± 0.6	2.2 ± 0.3
	D 7	11.37 ± 3.37	52.3 ± 15.6	43.2 ± 15.2	0.9 ± 0.4	0.6 ± 0.5	2.4 ± 0.6
	D 10	14.03 ± 0.90	47.6 ± 7.6	46.8 ± 6.2	0.8 ± 0.2	1.6 ± 0.9	2.6 ± 0.9
	D 14	12.54 ± 3.44	51.2 ± 12.9	44.6 ± 12.9	0.8 ± 0.2	0.5 ± 0.3	2.4 ± 0.8
	D 21	10.81 ± 4.39	30.2 ± 6.2	64.6 ± 8.7	0.4 ± 0.2	1.9 ± 2.3	2.3 ± 0.5
	D 28	8.84 ± 0.54	60.7 ± 16.9	33.7 ± 17.1	1.1 ± 0.5	1.0 ± 0.5	2.8 ± 0.7
EPO	D 0	11.96 ± 1.11	65.0 ± 3.6	27.6 ± 5.5	2.5 ± 2.3	1.7 ± 2.3	2.4 ± 0.6
	D 3	11.91 ± 1.47	51.5 ± 9.9	43.1 ± 10.1	1.1 ± 0.6	1.2 ± 0.8	2.3 ± 0.5
	D 7	7.82 ± 1.23	56.7 ± 7.9	34.2 ± 7.9	2.5 ± 2.4	1.4 ± 1.1	3.8 ± 0.5
	D 10	9.55 ± 1.64*	59.1 ± 4.6	34.6 ± 3.6	1.4 ± 1.5	4.2 ± 1.9	3.3 ± 0.2
	D 14	5.93 ± 0.46*	53.6 ± 5.8	37.6 ± 6.6	1.4 ± 1.9	2.1 ± 1.4	4.1 ± 0.3
	D 21	5.23 ± 0.76	36.1 ± 5.6	56.5 ± 3.8	0.9 ± 1.1	5.2 ± 2.1	4.2 ± 0.9
	D 28	4.71 ± 0.76*	64.5 ± 9.0	27.4 ± 5.6	1.4 ± 1.7	2.8 ± 1.6	4.1 ± 0.7

Each value represents as mean ± SD (n= 3).
Significant difference from each control group (* p < 0.05, ** p < 0.01).
LYM, lymphocyte; NEU, neutrophil; EOS, eosinophil; BAS, basophil; MON, monocyte

Changes in platelet-related parameters in cynomolgus monkeys

	Days	Platelet count (x1000/μl)	MPV	PDW	PCT
Control	D 0	332 ± 55	9.9 ± 0.7	61.8 ± 2.0	0.3 ± 0.0
	D 3	314 ± 42	9.8 ± 1.0	61.0 ± 3.6	0.3 ± 0.0
	D 7	311 ± 30	10.0 ± 0.8	61.2 ± 5.1	0.3 ± 0.0
	D 10	315 ± 44	10.4 ± 0.6	63.7 ± 1.4	0.3 ± 0.1
	D 14	291 ± 32	10.2 ± 0.3	66.5 ± 3.7	0.3 ± 0.0
	D 21	321 ± 28	10.0 ± 0.6	64.7 ± 4.3	0.3 ± 0.0
	D 28	208 ± 169	11.2 ± 1.5	66.6 ± 3.0	0.2 ± 0.2
EPO	D 0	380 ± 104	9.6 ± 1.3	70.4 ± 4.9	0.4 ± 0.1
	D 3	451 ± 102	9.7 ± 1.2	75.7 ± 10.9	**0.5 ± 0.1***
	D 7	452 ± 105	10.9 ± 2.4	86.8 ± 16.2	0.5 ± 0.1
	D 10	489 ± 112	11.5 ± 3.1	**89.9 ± 11.6***	0.6 ± 0.2
	D 14	421 ± 103	12.9 ± 4.5	**93.8 ± 5.3***	0.6 ± 0.3
	D 21	**534 ± 123***	12.5 ± 5.1	**91.9 ± 3.1***	0.7 ± 0.4
	D 28	437 ± 71	**13.9 ± 3.1**	**96.5 ± 5.0***	0.6 ± 0.2

Each value represents as mean ± SD (n= 3).
Significant difference from each control group (* p < 0.05, ** p < 0.01).
MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit

Changes in Serum biochemical values in cynomolgus monkeys (I)

	Day 7		Day 14		Day 21		Day 28	
	Control	EPO	Control	EPO	Control	EPO	Control	EPO
AST (IU/l)	37.3 ± 9.8	36.4 ± 11.3	38.9 ± 10.3	40.5 ± 23.5	45.8 ± 16.1	53.4 ± 18.9	40.8 ± 14.3	56.8 ± 12.2
ALT (IU/l)	41.7 ± 14.9	36.8 ± 9.4	43.5 ± 14.1	43.9 ± 9.7	45.9 ± 27.9	48.1 ± 10.6	52.4 ± 35.7	48.1 ± 15.6
ALP (IU/l)	1256.0 ± 410.5	1573.0 ± 470.6	1292.0 ± 433	1578.0 ± 333.6	1220.0 ± 327.5	1643.0 ± 329.1	1163.0 ± 390.6	1465.0 ± 349.1
BUN (mg/dl)	18.5 ± 2.0	19.1 ± 1.0	20.2 ± 2.0	17.5 ± 1.4	19.4 ± 2.8	17.0 ± 1.4	19.3 ± 2.2	16.6 ± 0.5
CREA (mg/dl)	1.1 ± 0.1	**0.8**** ± 0.0	1.1 ± 0.0	**0.8*** ± 0.2	1.0 ± 0.1	0.8 ± 0.0	1.0 ± 0.2	0.8 ± 0.0
GLU (mg/dl)	64.8 ± 5.8	49.5 ± 5.7	67.4 ± 5.5	52.7 ± 8.9	60.2 ± 7.6	**22.7**** ±10.5	66.1 ± 9.4	29.1 ± 10.3
T-CHO (mg/dl)	104.4 ± 25.8	110.7 ± 5.8	112.0 ± 24.0	119.0 ± 16.3	109.6 ± 12.9	123 ± 5.6	109.2 ± 17.9	121.0 ± 10.8
A/G (ratio)	1.75 ± 0.19	1.47 ± 0.10	1.69 ± 0.17	**1.36*** ± 0.09	1.69 ± 0.26	1.32 ± 0.07	1.67 ± 0.20	**1.29*** ± 0.06

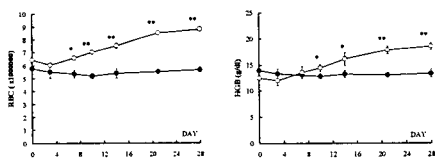
Each value represents as mean ± SD (n=3).
Significant difference from VC group (* p < 0.05, ** p < 0.01).

Changes in Serum biochemical values in cynomolgus monkeys (II)

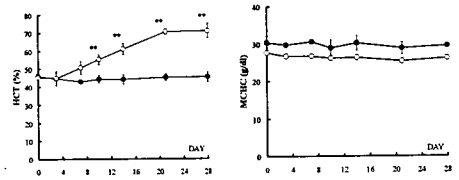
	Day 7		Day 14		Day 21		Day 28	
	Control	EPO	Control	EPO	Control	EPO	Control	EPO
TP (g/dl)	7.3 ± 0.2	**8.3*** ± 0.5	7.7 ± 0.4	**8.7*** ± 0.4	7.7 ± 1.0	9.1 ± 0.7	7.6 ± 0.6	8.7 ± 0.4
A/G (g/dl)	4.6 ± 0.1	4.9 ± 0.2	4.8 ± 0.1	5.0 ± 0.2	4.8 ± 0.3	5.2 ± 0.4	4.7 ± 0.2	4.9 ± 0.1
CPK (IU/l)	275.0 ± 89.8	303.0 ± 45.3	420.0 ± 88.0	424.0 ± 87.1	380.0 ± 115.7	410 ± 46.7	277.0 ± 115.1	452.0 ± 68.9
T-Bil (mg/dl)	0.16 ± 0.06	0.21 ± 0.05	0.19 ± 0.02	0.27 ± 0.08	0.18 ± 0.02	0.27 ± 0.03	0.18 ± 0.01	**0.28*** ± 0.05
IP (mg/dl)	4.7 ± 0.7	6.8 ± 0.7	4.9 ± 0.3	6.7 ± 0.3	4.1 ± 0.7	5.9 ± 0.5	5.0 ± 0.8	5.9 ± 0.6
Ca (mg/dl)	8.8 ± 0.5	9.0 ± 0.4	9.1 ± 0.3	9.2 ± 0.5	9.2 ± 0.5	9.7 ± 0.8	9.3 ± 0.4	9.5 ± 0.5
Cl (mmol/l)	113.0 ± 1.5	112.0 ± 0.1	107.0 ± 2.3	106.0 ± 2.1	106.0 ± 1.5	105.0 ± 2.3	105.0 ± 0.7	104.0 ± 3.8
Na (mmol/l)	146.0 ± 1.0	148.0 ± 1.0	147.0 ± 0.6	150 ± 1.5	147.0 ± 0.6	**152*** ± 1.7	149.0 ± 2.1	149.0 ± 0.6
K (mmol/l)	4.2 ± 0.2	4.5 ± 0.8	4.3 ± 0.7	4.5 ± 0.4	4.8 ± 0.8	6.0 ± 0.8	5.3 ± 0.1	4.6 ± 1.3

Each value represents as mean ± SD (n=3).
Significant difference from VC group (* p < 0.05, ** p < 0.01).

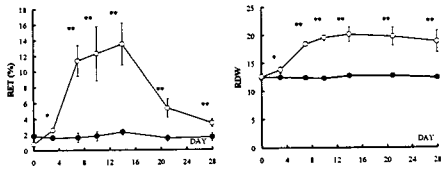
Changes in RBC-related parameters in cynomolgus monkeys treated with/without EPO (I)



Changes in RBC-related parameters in cynomolgus monkeys treated with/without EPO (II)



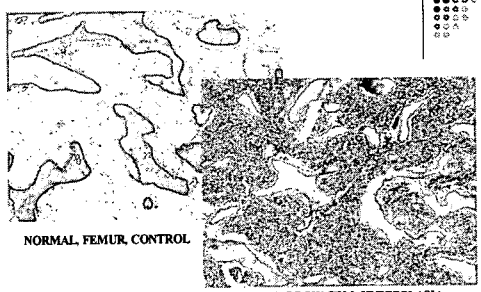
Changes in RBC-related parameters in cynomolgus monkeys treated with/without EPO (III)



Histopathological changes of target organs in cynomolgus Monkeys treated with/without EPO

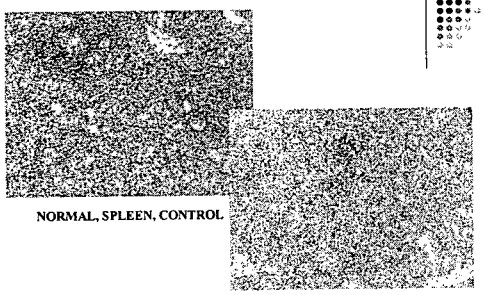
Organs	Vehicle Control	EPO
Kidney		
Congestion	2 (66.6)	3 (100)
Heart		
Congestion	2 (66.6)	3 (100)
Cardiomyopathy	1 (33.3)	1 (33.3)
Liver		
Ito cell hyperplasia	2 (66.6)	0 (0)
Inflammatory foci	0 (0)	2 (66.6)
Lung		
Congestion	0 (0)	1 (33.3)
Spleen		
Extramedullary hemopoiesis	0 (0)	**3 (100)**
Sternum		
Hyperplasia, bone marrow	0 (0)	**3 (100)**
Femur		
Hyperplasia, bone marrow	0 (0)	**2 (66.6)**
Testis		
Seminiferous tubular atrophy	0 (0)	0 (0)

- All tissues taken from each group (n= 3) at necropsy were examined microscopically.
- The other organs did not show any EPO-treated findings.



NORMAL, FEMUR, CONTROL

MARROW CELL HYPERPLASIA, FEMUR, TREATMENT



NORMAL, SPLEEN, CONTROL

EXTRAMEDULLARY HEMOPOIESIS, TREATMENT

Summary

1. The results indicated that rHuEPO treatment caused an increase in platelet- and RBC-related parameters, extramedullary hemopoiesis of spleen, and bone marrow hyperplasia of sternum and femur.

2. The present study will be valuable in the proper interpretation and validation of general toxicology studies for biogeneric drugs including rHuEPO in cynomolgus monkeys.

(Kim et al. 2005: J. Toxicol. Pub. Health 21: 227-234)

Key considerations for safety assessment using non-human primate

1. Why we need non-human primate for newly developed biotherapeutics
   - Closet animals relative to human
2. What we prepare in advance for a monkey study
   - Husbandry/Management : AAALAC, IACUC, Zoonoses
   - Experimental methodology : restraint, blood sampling technique, administration
   - Index of acclimatization for newly acquired monkey
     - Transport stress
     - Heavy metal level in blood
3. What we consider for safety assessment
   - Physiological levels in hematology, serum biochemistry, relative organ weight, and spontaneously histopathological lesions
   - Clinical signs (e.g., locomotion, circadian rhythm of locomotion)
   - Effect of ketamine anesthesia on biological variation in blood parameter

→ Key considerations will offer good information on safety/efficacy evaluation at early stage of the newly developed drug including biotherapeutics.

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