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**THE INFLUENCE OF SINGLE OR REPEATED PHLEBOTOMY
ON THE PHYSIOLOGICAL CONDITION OF
NORMAL AND DISEASED RATS**

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Accepted September 10, 1986

Abstract.....The present experiment was performed to see the influence of phlebotomy on hematology, serum chemistry, and pathology data in rats. When normal rats received a single phlebotomy of 2 or 3 ml blood, erythrocyte count and hematocrit were slightly reduced along with a persistent depression of the blood pressure. In repeated phlebotomy studies, 0.25 to 2 ml each of blood was removed from each rat every other day for 10 days. Normal rats from which 0.5 ml and more of blood was withdrawn showed decreases in erythrocyte count and serum iron level concomitant with an increase in reticulocyte count. Similar findings were obtained in rats with CCl₄ liver damage or gentamicin nephrotoxicity, except that those rats hardly exhibited anemia because of a simultaneous occurrence of hemoconcentration. Histopathological findings of the liver and kidney were not different between animals with and without phlebotomy. In phenylhydrazine-treated rats receiving repeated phlebotomy, one rat in the 1-ml phlebotomy and two rats in the 2-ml phlebotomy died from severe anemia. Surviving rats receiving 2-ml phlebotomy showed higher serum GOT and GPT activities together with fatty deposition and/or focal necrosis of hepatocytes. It is concluded that every-other-day phlebotomy of up to 0.25 ml each does not significantly influence the physiological condition of normal or diseased rats.

Key words: rat, phlebotomy, carbon tetrachloride, gentamicin sulfate, phenylhydrazine hydrochloride.

INTRODUCTION

It is important to assess the fluctuation of hematological and biochemical parameters periodically in drug safety studies. However, repeated blood sampling from a number of conscious rats is quite difficult. Thus, we have previously developed a practical technique for the collection of blood from the tail vein of the rat (Furuhama and Onodera, 1983). The present study was designed to investigate whether repeated phlebotomy could affect hematology, serum chemistry, and pathology data in normal rats and in rats in diseased states produced by conditions such as carbon tetrachloride (CCl_4) liver damage, gentamicin sulfate (GM) renal damage, or phenylhydrazine hydrochloride (PHZ) anemia. Because published investigations on this species are few, this study will enhance the value of the body of reference.

MATERIALS AND METHODS

Six to seven week-old male Sprague-Dawley rats (Clea Japan Inc., Tokyo), weighing 180g to 220g, were used throughout the studies. The animals were given a standard diet and a drinking water *ad libitum* and kept at a temperature of $23 \pm 2^\circ\text{C}$, with $55 \pm 10\%$ relative humidity and a controlled 12-hr light cycle. Groups of 5 normal rats each received a single phlebotomy of 0.25, 0.5, 1, 2, or 3 ml per animal or repeated phlebotomy of 0.25, 0.5, 1, or 2 ml per animal every other day (Days 1, 3, 5, 8, and 10) for 10 days. Experimental hepatic damage was produced in rats by administering orally with 0.5ml/kg of carbon tetrachloride (CCl_4) diluted with a ten-fold volume of olive oil every other day for 10 days. Experimental renal damage were induced in rats by subcutaneous injection of 80mg/kg of gentamicin sulfate (GM) once daily for 10 consecutive days. Experimental anemia were produced in rats by subcutaneous injection of 30mg/kg of phenylhydrazine hydrochloride (PHZ) 3 times (Days 1, 3, and 5) for 5 days. CCl_4 - and GM-treated rats received repeated phlebotomy just as described for normal rats, while for PHZ-treated rats phlebotomy was repeated twice (Days 2 and 4) because of the occurrence of death during the study. The phlebotomy procedure was the same method as previously reported (Furuhama and Onodera, 1983). Each animal was sacrificed by exsanguination 24 hrs after the last phlebotomy. Erythrocyte count (Coulter counter method), hematocrit (microhematocrit centrifugation method), and reticulocyte count (new methylene blue stain) were determined along with measurements of serum glutamic-oxaloacetic transaminase (GOT : enzyme method), glutamic-pyruvic transaminase (GPT : enzyme method), urea nitrogen (UN : urease-indophenol method), creatinine (Jaffe method), iron (bathophenanthroline disulfonic acid method), and copper (Landers-Zak method). Samples of the liver, kidney, and spleen were fixed in buffered neutral formalin, embedded in paraffin wax, sectioned at $5 \mu\text{m}$, and stained with hematoxylin

and eosin for histopathological examination. Berlin blue stain was also employed. Smear preparations of the femoral bone marrow obtained from all the animals were made with subsequent staining (Wright-Giemsa stain) and examination of the granulopoietic per erythropoietic (G/E) ratio. In one single phlebotomy study, blood pressure was also measured using the tail cuff method under the conscious condition 5 min, 3 hr, 6 hr, and 24 hr after phlebotomy.

The results were statistically analyzed by Student's *t* test for comparison between control and treated groups.

RESULTS

1. Single phlebotomy

There were no changes in hematological parameters at 0.25, 0.5, or 1ml phlebotomy. Phlebotomy of 2 ml or more caused decreases in erythrocyte count and hematocrit, and the 3-ml phlebotomy additionally caused a slight increase in reticulocyte count (Table 1). Blood pressure were reduced by 12% in the 1-ml phlebotomy, by 21% in the 2-ml phlebotomy, and by 32% in the 3-ml phlebotomy as compared with that at pre-phlebotomy (132 ± 2 mmHg; *n* = 25). Regarding the reversibility of the blood pressure reduction, the values returned to the baseline level at 6 hr after phlebotomy treatment of up to 2 ml, but not even after 24 hr in the 3-ml phlebotomy (Fig. 1). No changes considered to be related to the single phlebotomies were seen in the liver, kidney, or spleen.

Table 1 Clinical values in normal rats that underwent a single phlebotomy

Group	N	RBC ($\times 10^6/\mu\text{l}$)	Ht (%)	Retics (% RBC)	G/E ratio	Serum			
						GPT (IU)	Creatinine (mg/dl)	Fe ($\mu\text{g}/\text{dl}$)	Cu ($\mu\text{g}/\text{dl}$)
No treatment	5	6.24 ± 0.19	43.6 ± 1.0	5.8 ± 0.6	1.31 ± 0.10	17 ± 2	0.93 ± 0.11	157 ± 8	101 ± 2
0.25 ml ^a	5	6.27 ± 0.17	43.6 ± 0.4	5.3 ± 0.4	1.27 ± 0.10	17 ± 4	0.90 ± 0.12	165 ± 11	94 ± 5
0.5 ml	5	5.66 ± 0.09	41.3 ± 0.8	5.5 ± 0.7	1.24 ± 0.08	20 ± 3	0.96 ± 0.06	159 ± 16	107 ± 4
1 ml	5	5.86 ± 0.28	40.5 ± 0.5	5.5 ± 0.7	1.25 ± 0.10	20 ± 1	0.77 ± 0.04	186 ± 2	94 ± 4
2 ml	5	5.38 $\pm 0.20^*$	37.1 $\pm 1.6^*$	7.7 ± 0.3	1.22 ± 0.07	17 ± 3	0.90 ± 0.10	188 ± 19	103 ± 3
3 ml	5	4.96 $\pm 0.07^*$	35.6 $\pm 1.6^*$	7.9 $\pm 0.4^*$	1.06 ± 0.08	18 ± 6	0.81 ± 0.12	216 ± 32	106 ± 5

Clinical tests were performed 24 hrs after phlebotomy.

* : Significant difference from non-treated group ($P < 0.05$).

^a : Blood volume phlebotomised.

Fig. 1. Blood pressure (B. P.) in normal rats that underwent a single phlebotomy. Mean B. P. at pre-treatment was 132 ± 2 mmHg ($n=25$) which was expressed as 100%.
 α : Blood volume phlebotomised.

2. Repeated phlebotomy

Normal rats from which 0.5 ml or more of blood was removed showed decreases in erythrocyte count and serum iron level concomitant with an increase in reticulocyte count. Additionally, rats receiving the 2-ml phlebotomy showed slight reduction of the G/E ratio (Table 2). In the terminal examination, slight increase in the number of erythroblasts was observed in the red pulp of the spleen in the 2-ml phlebotomy. No noticeable changes attributable to repeated phlebotomy were observed in the liver or kidney.

CCl_4 -treated rats showed tendencies of hemoconcentration and elevation in the serum activities of GOT and GPT, together with fatty infiltration and ballooning in the centrilobular hepatocytes. After repeated phlebotomy, there were a slight increase in the reticulocyte count and a decrease in the G/E ratio in the 0.5-ml phlebotomy or more, and additional decreases in erythrocyte count and hematocrit in the 2-ml phlebotomy (Table 3). No potentiation of hepatotoxicity by repeated phlebotomy was detected functionally or morphologically among the phlebotomy groups.

GM-treated rats showed hemoconcentration, decrease in the reticulocyte count, an increase in the G/E ratio, elevation in the serum levels of UN and creatinine, extensive necrosis and degeneration of the proximal tubular epithelia, and hyaline cast in the tubular lumens. Rats undergoing 1 ml or greater phlebotomies showed

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Table 2 Clinical values in normal rats that underwent repeated phlebotomy

Group	N	RBC ($\times 10^6/\mu\text{l}$)	Ht (%)	Retics (% RBC)	G/E ratio	Serum			
						GPT (IU)	Creatinine (mg/dl)	Fe ($\mu\text{g}/\text{dl}$)	Cu ($\mu\text{g}/\text{dl}$)
No treatment	5	7.35 ± 0.16	49.9 ± 1.5	3.8 ± 0.2	1.51 ± 0.23	18 ± 6	0.67 ± 0.14	184 ± 36	92 ± 2
0.25 ml ^a	5	7.02 ± 0.19	47.4 ± 1.2	4.2 ± 0.3	1.55 ± 0.21	27 ± 5	0.68 ± 0.14	136 ± 14	101 ± 8
0.5 ml	5	6.84 $\pm 0.11^*$	47.4 ± 1.1	5.6 $\pm 0.4^*$	1.51 ± 0.18	22 ± 6	0.70 ± 0.18	104 $\pm 10^*$	90 ± 3
1 ml	5	6.33 $\pm 0.10^*$	46.3 ± 0.5	7.3 $\pm 0.8^*$	1.51 ± 0.06	27 ± 1	0.71 ± 0.03	117 ± 24	107 ± 10
2 ml	5	5.72 $\pm 0.15^*$	43.1 $\pm 1.0^*$	15.0 $\pm 0.3^*$	0.91 $\pm 0.16^*$	25 ± 3	0.59 ± 0.12	65 $\pm 15^*$	103 ± 3

Phlebotomy was repeated every other day (Days 1, 3, 5, 8, and 10) for 10 days and clinical tests were performed on Day 11.

* : Significant difference from non-treated group ($P < 0.05$).

^a : Blood volume phlebotomised.

Table 3 Clinical values in CCl₄-treated rats that underwent repeated phlebotomy

Group	N	RBC ($\times 10^6/\mu\text{l}$)	Ht (%)	Retics (% RBC)	G/E ratio	Serum		
						GOT (IU)	GPT (IU)	Fe ($\mu\text{g}/\text{dl}$)
CCl ₄ + no phlebotomy	5	6.51 ± 0.13	44.8 ± 0.7	4.4 ± 0.5	1.23 ± 0.08	498 $\pm 72^*$	225 $\pm 15^*$	151 ± 13
CCl ₄ + 0.25 ml ^a	5	6.33 ± 0.11	44.8 ± 1.1	5.4 ± 0.3	1.08 ± 0.06	553 $\pm 130^*$	224 $\pm 68^*$	150 ± 14
CCl ₄ + 0.5 ml	5	6.31 ± 0.15	44.9 ± 0.8	6.6 $\pm 0.6^{**}$	1.00 $\pm 0.05^*$	668 $\pm 107^*$	369 $\pm 120^*$	126 ± 18
CCl ₄ + 1 ml	5	5.93 ± 0.21	43.5 ± 0.9	8.5 $\pm 0.6^{**}$	0.92 $\pm 0.03^{**}$	466 $\pm 133^*$	235 $\pm 97^*$	145 ± 25
CCl ₄ + 2 ml	5	5.15 $\pm 0.12^{**}$	40.3 $\pm 0.9^{**}$	19.6 $\pm 1.1^{**}$	0.75 $\pm 0.07^{**}$	464 $\pm 52^*$	198 $\pm 33^*$	241 ± 40
No treatment	5	6.23 ± 0.12	43.4 ± 0.4	4.5 ± 0.2	1.18 ± 0.12	128 ± 19	35 ± 4	156 ± 25

Animals were treated by gavage with 0.5 ml/kg of CCl₄ every other day for 10 days.

Phlebotomy was repeated every other day (Days 1, 3, 5, 8, and 10) for 10 days and clinical tests were performed on Day 11.

* : Significant difference from CCl₄-treated rats without phlebotomy ($P < 0.05$).

** : Significant difference from non-treated group ($P < 0.05$).

^a : Blood volume phlebotomised.

decreased erythrocyte count and hematocrit (Table 4). No potentiation of renal toxicity was seen due to repeated phlebotomy.

PHZ-treated rats developed severe anemia characterized by decreases in erythrocyte count, hematocrit, and G/E ratio, and marked increase in reticulocyte count (Table 5). Histopathological examination of the spleen revealed congestion, increased number of erythroblasts, and enhancement of hemosiderin deposits in the red pulp. Hemosiderin was also contained in the proximal tubular epithelia of the kidney and swollen Kupffer cells of the liver. The Kupffer cells simultaneously showed erythrophagia. One rat of the 1-ml phlebotomy group and two rats of the 2-ml phlebotomy group died. Two of the 3 surviving rats undergoing the 2-ml the phlebotomy showed higher serum GOT and GPT activities together with fatty deposition and/or focal necrosis of the hepatocytes (Photo. 1). No changes were found in the liver or kidneys of the three dead rats.

Table 4 Clinical values in gentamicin sulfate (GM)-treated rats that underwent repeated phlebotomy

Group	N	RBC ($\times 10^6/\mu\text{l}$)	Ht (%)	Retics (% RBC)	G/E ratio	Serum		
						UN (mg/dl)	Creatinine (mg/dl)	Fe ($\mu\text{g}/\text{dl}$)
GM + no phlebotomy	5	7.40 $\pm 0.28^*$	44.7 $\pm 2.2^*$	0.1 $\pm 0.1^*$	1.76 $\pm 0.34^*$	130 $\pm 15^*$	5.25 $\pm 1.19^*$	178 ± 15
GM + 0.25 ml ^a	5	6.46 $\pm 0.16^*$	38.9 $\pm 1.4^*$	0.3 $\pm 0.1^*$	2.41 $\pm 0.24^*$	123 $\pm 4^*$	3.91 $\pm 0.32^*$	165 ± 17
GM + 0.5 ml	5	6.55 $\pm 0.13^*$	40.4 $\pm 0.6^*$	0.1 $\pm 0.1^*$	2.13 ± 0.30	96 $\pm 20^*$	3.46 $\pm 0.85^*$	150 ± 9
GM + 1 ml	5	5.72 $\pm 0.25^{**}$	36.2 $\pm 1.2^{**}$	0.1 $\pm 0.1^*$	1.99 $\pm 0.10^*$	110 $\pm 15^*$	3.50 $\pm 0.87^*$	167 ± 10
GM + 2 ml	5	4.94 $\pm 0.18^{**}$	28.5 $\pm 1.8^{**}$	2.1 ± 0.7	1.53 ± 0.26	111 $\pm 19^*$	3.22 $\pm 0.67^*$	153 ± 7
No treatment	5	6.69 ± 0.18	41.5 ± 1.4	5.0 ± 0.7	1.29 ± 0.10	18 ± 1	0.53 ± 0.05	156 ± 8

Animals were treated subcutaneously with 80 mg/kg of GM once daily for 10 days.

Phlebotomy was repeated every other day (Days 1, 3, 5, 8, and 10) and clinical tests were performed on Day 11.

* : Significant difference from GM-treated rats without phlebotomy ($P < 0.05$).

** : Significant difference from non-treated group ($P < 0.05$).

^a : Blood volume phlebotomised.

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Table 5 Clinical values in phenylhydrazine hydrochloride (PHZ)-treated rats that underwent repeated phlebotomy

Group	N	RBC ($\times 10^6/\mu\text{l}$)	Ht (%)	Retics (% RBC)	G/E ratio	Serum			
						GOT (IU)	GPT (IU)	Creatinine (mg/dl)	Fe ($\mu\text{g}/\text{dl}$)
PHZ + no phlebotomy	5	3.42 $\pm 0.10^*$	33.1 $\pm 1.2^*$	90.5 $\pm 8.3^*$	0.59 $\pm 0.01^*$	173 ± 9	24 ± 3	0.70 ± 0.02	135 $\pm 3^*$
PHZ + 0.25 ml ^a	5	3.32 $\pm 0.14^*$	32.9 $\pm 1.0^*$	100.5 $\pm 10.1^*$	0.76 $\pm 0.10^*$	134 ± 12	21 ± 2	0.79 ± 0.06	130 $\pm 20^*$
PHZ + 0.5 ml	5	3.19 $\pm 0.10^*$	34.1 $\pm 1.5^*$	127.1 $\pm 11.3^*$	0.62 $\pm 0.04^*$	173 ± 11	27 ± 7	0.82 $\pm 0.03^{**}$	121 $\pm 8^*$
PHZ + 1 ml	5	3.02 $\pm 0.04^{**}$	33.0 $\pm 0.7^*$	136.4 $\pm 13.5^*$	0.72 $\pm 0.11^*$	168 ± 13	45 ± 9	0.80 $\pm 0.03^{**}$	149 $\pm 19^*$
PHZ + 2 ml	5	2.71 $\pm 0.25^{**}$	25.8 $\pm 4.3^*$	—	0.70 $\pm 0.08^*$	320 ± 136	92 ± 52	1.01 ± 0.07	137 $\pm 14^*$
No treatment	5	6.96 ± 0.27	48.9 ± 1.6	3.6 ± 0.5	0.92 ± 0.08	129 ± 12	22 ± 3	0.62 ± 0.06	215 ± 10

Animals were treated subcutaneously with 30 mg/kg of PHZ every other day (Days 1, 3, and 5) for 5 days. Phlebotomy was repeated twice (Days 2 and 4) for 5 day and clinical tests were performed on Day 6.

* : Significant difference from PHZ-treated rats without phlebotomy ($P < 0.05$).

** : Significant difference from non-treated group ($P < 0.05$).

- : No detectable for cell debris.

^a : Blood volume phlebotomised.

Photo. 1 Liver from PHZ-treated rat that underwent 2 ml phlebotomy. H. E. staining $\times 170$, Note fatty deposition and focal necrosis of hepatocytes.

DISCUSSION

The loss of blood from the body reduces the blood pressure and may interfere with the constituents of the remaining blood (Todd and Ross, 1968). The aim of this investigation was to assess the influence of phlebotomy in normal and diseased rats. When normal rats underwent a single phlebotomy of 2 or 3 ml blood, erythrocyte count and hematocrit were slightly reduced along with a persistent depression of the blood pressure. In repeated phlebotomy studies, 0.25 to 2 ml of blood was removed from each rat every other day for 10 days. Normal rats from which 0.5 ml or more of blood was withdrawn showed decreases in erythrocyte count and serum iron level with increase in reticulocyte count. These results were quite similar to those of previous studies (Furuhama and Onodera, 1983). Decreased serum iron level was a change which did not occur in the single phlebotomy study. It might be considered that the availability of iron for hematopoiesis was accelerated by repeated loss of blood. Serum copper levels and histopathological findings of the liver and kidney showed no abnormalities. CCl₄- and GM-treated rats seemed to hardly exhibit anemia because of a simultaneous occurrence of hemoconcentration. It has been reported that the high dose treatment of CCl₄ or GM to rats produces hemoconcentration but not anemia (Tobe et al., 1981 ; Hara, 1985). The cause of hemoconcentration due to these compounds might be considered to be related to the dehydration resulting from decreased food and water intakes, or in the case of GM from increased urinary volume. Anemia due to phlebotomy is usually accompanied by an increase in reticulocyte count, but in these rats the reticulocyte count was rather reduced probably because erythropoietic response was lowered under the hemoconcentrated condition. Histopathological findings of the liver and kidney in CCl₄- or GM-treated rats were unaffected by repeated phlebotomy. In PHZ-treated rats that underwent repeated phlebotomy, one rat in the 1-ml phlebotomy and two rats in the 2-ml phlebotomy died from severe anemia. The surviving rats undergoing the 2-ml phlebotomy exhibited liver damage including elevated serum activities of GOT and GPT, fatty deposition, and/or focal necrosis of hepatocytes. The anemic effect of PHZ has been widely investigated (Beaven and White, 1954 ; Onodera et al., 1985). Furthermore, PHZ and other hydrazine derivatives are known to affect various proteins and enzymes of the liver (Asami, 1968 ; Pankow and Ponsold, 1976). It is interesting to note that repeated phlebotomy produced a significant enhancement in the hepatotoxic potentials of PHZ. This finding suggests that bleeding of rats with anemia must be performed with caution from the point of toxicological evaluation.

The results presented above show that every-other-day phlebotomy of up to 0.25 ml each time did not significantly influence the physiological condition of normal or diseased rats.

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