大韓獸醫學會誌(1993) 第33券 第3號
Korean J Vet Res (1993) 33(3) : 507~511

The effect of captafol on the hematological value, erythrocyte membrane and plasma biochemical value *in vitro*

Sung-hoon Kim, Hwa-seon Hwang, Shin-woo Cha,
Sang-seop Han, Jung-koo Roh
Toxicology Research Center Korea Research Institute of Chemical Technology
(Received June 16, 1993)

시험관내에서 captafol이 혈액학, 적혈구막 및 혈장생화학치에 미치는 영향

김성훈 · 황화선 · 차신우 · 한상섭 · 노정구
한국화학연구소 안전성연구센터
(1993년 6월 16일 접수)

초록 : Captafol은 phthalimide계통의 항진균제로써 difolatan이란 상품명으로 개발되어 과일, 채소의 탄저병 및 원목 등의 곰팡이 구제에 널리 사용되고 있는 것으로 국내에서도 두번째로 많이 생산, 사용되는 품목이나 독성실험에 관한 보고는 거의 없는 상태이다. 이에 본 연구에서는 captafol 자체에 대한 독성기전의 일부를 확인하고자 동물체내에서 일어나는 여러가지 대사작용이 배제된 시험관내에서 실험을 수행하였다.

랫트 혈액을 후대정맥으로 부터 채혈하여 4개의 시험관에 2.5㎖씩 분주한 후 captafol 농도가 0.1×10^{-4}M, 1×10^{-3}M, 1×10^{-2}M이 되도록 한 ethanol용액 25㎕ 씩 넣고 CO_2 incubator에서 2시간동안 부드럽게 혼합하여 혈액학적 성상 및 적혈구 취약성 시험을 수행하였다. 또한 원심분리하여 얻어진 혈장에서 생화학적 성상 등을 조사한 결과 다음과 같은 성적을 얻었다.

1. 적혈구수 및 hematocrit치는 captafol 투여량에 비례적으로 유의한($p<0.05$) 감소를 나타내었다.
2. 적혈구막 취약성 실험에서 1×10^{-3}M 및 1×10^{-2}M 농도에서 대조군에 비하여 유의한($p<0.01$) 증가를 나타내었다.
3. 혈장내 potassium이온의 농도는 captafol의 투여량에 비례적으로 증가하였다.
4. Total bilirubin 및 creatine kinase가 대조군에 비하여 각각 증가 및 감소하였다.

Key words : captafol, hematological value, erythrocyte membrane, plasma biochemical value, *in virto*.

Introduction

Captafol [3a, 4, 7, 7a-Tetrahydor-2-1, 1,2,2-tetrachloroethylthio-1H-isoindole-1,3(2H)-dion] is one of the widely used fungicide in Korea. The fungitoxic action of trichloromethylthio fungicide has been extensively studied and suggested to be due to an affinity of the chemical for sulfhydryl groups.[1~6] Lukens[7~9] has postulated the mechanism of action of this fungicide as follow. When RN-$SCCl_3$ compounds react with cell thiols, the fungicide split at the RN-S bond, forming the free imide, and the free thiol is oxidized to disulfide. The liberated trichloromethylthio($SSCl_3$) moiety can be transferred directly to various cellular sites or yield thiophosgene which may

react with thiols and other cellular groups. Lukens has suggested that captafol enters reaction in addition to the oxidation of thiols.

Since sulfhydryl groups are known to play an importa-nt role in maintaining the structural integrity of cell membrane,[10] it is quite likely that captafol, due to its high affinity for sulfhydryl groups, may interfere with membrane sturcture and function.[11]

It was assumed that the shortened survival time of erythrocyte due to the decreased concentration of phospholipid and the increment of permeability with captafol might be attributed to the functions of the membrane structural substances. The phthalimide of captafol is reacted with thiol of creatine kinase subunit. Creatine kinase is decreased and total bilirubin level due to the shortened survival time of erythrocyte is increased. Therefore, this experiment was studied in order to characterize the effect of erythrocyte membrane, hematological and plasma biochemical values by captafol *in vitro*.

Materials and methods

Blood was collected from vena cava of 5 SPF Sprague-Dawley male rats(10 weeks), using 0.5mg of heparin(approx. 170 USP unit/mg) per 10mℓ of blood as the anticoagulant. The blood was preincubated for 10min. before the addition of the captafol solutions. Three concentrations(1×10^{-4}M, 1×10^{-3}M and 1×10^{-2}M) of captafol in ethanol were added to 2.5mℓ of blood so that the final concentration of ethanol was 1%.[11] After the addition of the test chemical, the blood was incubated at 37℃ for 2 hours under 5% CO_2 gas. Control blood were added with similar concentration of ethanol : 1% ethanol.

After incubation, leucocyte count (WBC), erythrocyte count(RBC), hemoglobin concentration (Hgb), hematocrit (Hct), mean corpuscular volume(MCV), mean corpuscular hemoblobin(MCH) and mean corpscular hemoglobin concentration (MCHC) were determined by a hematological autoanalyzer(Coulter S 880, USA).

The blood was examined for erythrocyte membrane fragility test by the method of Gordon et al.[12]

The blood was centrifuged at 1000g for 10min. The plasma was collected by aspiration. Plasma biochemistry was examined to quantify plasma levels of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRN), glucose (GLU), total cholesterol (TCHO), total protein (TP), albumin (ALB), total bilirubin (TBIL), phospholipid (PL), triglyceride (TG), creatine kinase (CPK) and calcium (CA) by biochemical autoanalyzer (JCA VX-1000, Jeol Co). and potassium (K) by flame photometer(IL 943, Instrumentation Laboratory).

For control and treatment group statistical significance was tested using the Student t-test. A difference was considered statistically significant at $p<0.05$ and $p<0.01$.

Results

The effect of captafol on the hematological parameters of male rats are shown in Table 1. The number of RBC in only 1×10^{-2}M group was significantly($p<0.05$) decreased to 6.78 ± 0.28 millions compared to the control group (7.27 ± 0.41 millions). The percentage of Hct was significantly ($p<0.05$) decreased with dose-response at dose levels of 1×10^{-4}M($41.35\pm1.66\%$), 1×10^{-3}M($41.15\pm1.41\%$), 1×10^{-2}M($39.52\pm3.14\%$) compared with the control group($44.93\pm3.14\%$).

The concentration of MCHC in 1×10^{-3}M group (35.70 ± 0.47 g/dℓ) was significantly ($p<0.05$) increased from that of control (32.63 ± 2.94g/dℓ), but 1×10^{-2}M group, as a high dose, was not significant.

The effects of captafol on the erythrocyte membrane fragilities of male rats are shown in Table 2. Erythrocyte fragility rates of control 1×10^{-4}M, 1×10^{-3}M and 1×10^{-2}M groups were $2.64\pm1.46\%$, $2.99\pm1.27\%$, $4.85\pm2.13\%$ and $5.27\pm1.46\%$, respectively. Fragility rates of the 1×10^{-3}M and 1×10^{-2}M group were significantly ($p<0.01$) increased from control value with a dose-response.

The effect of captafol on the plasma potassium ion of the male rats are shown in Table 3. Potassium ion levels of control, 1×10^{-4}M, 1×10^{-3}M and 1×10^{-2}M groups were 4.95 ± 0.15 mmol/ℓ, 5.11 ± 0.35 mmol/ℓ, 5.13 ± 0.22 mmol/ℓ and 5.53 ± 0.32 mmol/ℓ, respectively. Although there was a dose-response, potassium ion level of 1×10^{-2}M group was significantly($p<0.05$) increased from that of control. However, the other groups were not significant.

The effect of captafol on the plasma biochemical

Table 1. Effect of captafol on the hematological parameters of male rats

	Control	1×10^{-4}M	1×10^{-3}M	1×10^{-2}M
WBC(thousands)	16.77±3.52	15.88±4.62	16.80±6.18	16.07±4.58
RBC(millions)	7.27±0.41	6.99±0.35	6.94±0.30	6.78±0.28*
Hgb(gdℓ)	14.58±0.59	14.57±0.63	14.67±0.59	14.70±0.64
Hct(%)	44.93±3.14	41.35±1.66*	41.15±1.41*	39.52±3.39*
MCV(fℓ)	61.92±4.88	59.17±1.11	59.28±1.02	58.17±3.29
MCH(pg)	20.10±1.30	20.83±0.59	21.17±0.37	21.67±1.35
MCHC(g/dℓ)	32.63±2.94	35.27±0.71	35.70±0.47*	37.52±4.76

*: Significantly different from control value at $p<0.05$.

Table 2. Effect of captafol on the erythrocyte membrane fragilities of male rats(%)

ID NO.	Control	1×10^{-4}M	1×10^{-3}M	1×10^{-2}M
1	0.64	3.49	4.72	4.55
2	3.46	1.37	7.50	7.50
3	1.80	4.40	8.35	4.55
4	2.89	2.69	6.65	3.16
5	1.56	4.24	3.09	3.56
6	0.00	2.57	5.15	4.74
7	2.75	0.00	1.33	5.31
8	2.05	3.86	5.30	4.55
9	3.84	3.72	2.06	5.46
10	3.61	2.92	2.07	7.56
11	3.84	4.17	6.04	7.58
12	3.64	2.28	4.66	3.81
13	3.79	2.05	6.67	6.19
14	3.03	4.09	4.29	5.24
Mean±SD	2.64±1.23	2.99±1.27	4.85±2.13**	5.27±1.46**

**: Significantly different from control value at $p<0.01$.

Table 3. Effect of captafol on the plasma potassium ion of male rats(mmol/ℓ)

ID NO.	Control	1×10^{-4}M	1×10^{-3}M	1×10^{-2}M
1	4.74	5.01	5.04	5.24
2	5.15	4.99	5.34	5.80
3	5.06	4.91	4.92	5.96
4	4.83	5.77	5.22	5.59
5	5.00	5.20	5.39	5.41
6	4.93	4.79	4.86	5.15
Mean±SD	4.95±0.15	5.11±0.35	5.13±0.22	5.53±0.32**

**: Significantly different from control value at $p<0.01$.

values of the male rats are shown in Tabel 4. The concentrations of TBIL in the 1×10^{-3}M and 1×10^{-2}M groups were significantly increased from that of control. The enzyme level of CPK in high dose group was significantly($p<0.05$) decreased from that of control. However, no treatment-related changes in the GOT, GPT, ALP, BUN, CRN, GLU, TCHO, TP, ALB, PL, TG and CA were observed under this experimental conditions.

Discussion

The findings in this *in vitro* study supports the *in vitro* observation of Kumer's study that captafol alters the erythrocytes permiability to ion. Captafol is known to react readily with cellular thiol groups.[1~6] The dichloroethylthioacylchloride (S-CCl_2-$CHCl_2$) moieties produced as a result of the reaction of thiols with captafol is also captafol of reaction with sulfhydryl and amino groups. The oxidation of thiols and the subsequent reactivity of th-

Table 4. Effect of captafol on the plasma biochemical values of the male rats.

	Control	1×10^{-4}M	1×10^{-3}M	1×10^{-2}M
GOT(IU/ℓ)[1]	104.38±6.34	100.46±6.95	100.51±8.24	102.40±5.20
GPT(IU/ℓ)[2]	77.00±11.20	72.56±6.22	74.891±0.49	71.50±6.64
ALP(IU/ℓ)[3]	618.51±142.61	565.53±98.11	578.88±160.41	561.09±107.99
BUN(mg/dl)[4]	18.12±2.91	17.80±1.93	17.94±2.92	17.87±2.11
CRN(mg/dℓ)[5]	0.48±0.04	0.51±0.03	0.50±0.06	0.52±0.11
GLU(mg/dℓ)[6]	150.82±10.84	159.18±8.98	151.53±17.40	150.80±11.34
TCHO(mg/dℓ)[7]	101.80±6.46	101.12±7.12	106.21±7.14	105.25±5.79
TP(g/dℓ)[8]	7.03±0.12	6.97±0.19	7.98±2.63	7.91±3.40
ALB(g/dℓ)[9]	5.27±0.98	5.60±0.19	5.61±0.38	5.57±0.33
TBIL(g/dℓ)[10]	2.37±0.37	2.51±0.26	3.10±0.30**	2.91±0.14*
PL(mg/dℓ)[11]	165.10±11.36	157.97±11.54	165.35±10.87	162.89±8.34
TG(mg/dℓ)[12]	211.67±58.78	222.52±60.02	213.01±64.22	219.41±72.18
CPK(IU/ℓ)[13]	315.77±79.14	280.88±30.54	260.38±51.94	219.41±29.63*
CA(mg/dℓ)[14]	9.74±0.53	10.00±0.46	9.76±0.57	9.41±1.19

*: Significantly different from control value at $p<05$.

**: Significantly different from control value at $p<0.01$.

[1][2][13]UV rate [3]P-Npp [4]urease-uv[5]jaffe [6][7][11]enzyme [8]biuret [9]BCG [10]jendrassik-cleghorn [12]GPO enzyme [14]OCPC.

e $SCCl_2$ moiety and of thiophosgene have been postulated to be the basis for toxicity of this fungicide.[1~6] Sulfhydryl groups are known to play an importat role in maintaining the structural integrity of the erythrocyte membrane.[13] Rothstein et al.[14] suggested that the blockage of cell membrane SH groups by sulfhydryl reagents after the cation permeability. It is, therefore, likely that the reaction of captafol and/or it's reaction products with the SH groups in the cell membrane is responsible for the observed changes in cation permeability. Sutherland et al.[15] suggested that SH reagents react with the sulfhydryl groups of the membrane protein resulting in a change in its configuration, causing a loss of ion from the cell. Kumer et al.[11] and findings in this study that the captafol reacted with a substantial portions of the membrane SH groups and potassium ion levels of plasma was increased by captafol, respectively, provide support for these hypothesis.

We concluded that the captafol at concentrations over 1×10^{-3}M induced the damage of erythrocyte membrane following the captafol was effective in causing the efflux of intracellular potassium ion, increment of erythrocyte membrane fragility rate and decrement of hematocrit value. We assumed that the phospholipid was efflux from the erythrocyte membrane, it is not a portion but analyzed products of the phospholipid by the captafol.

Creatine kinase contains one thiol group for each subunit.[16] The enzyme is rapidly inactivated by oxidation of these sulfhydryl group.[17] We assumed that, because the phthalimide of captafol was reacted with thiols of erythrocyte cell, creatine kinase was decreased with dose-response and distruction of erythrocyte was activated the increment of total bilirubin.

Summary

The effect of captafol on the hematological value, erythrocyte membrane and plasma biochemical value was investigated using blood of SPF Sprague-Dawley male rats *in vitro*. For the anticoagulations, we used 0.5mg of heparin per 10mℓ of blood from vena cava. Three concentrations(1×10^{-4}M, 1×10^{-3}M and 1×10^{-2}M) captafol in ethanol were added to the each 2.5mℓ blood so that the final concentration of ethanol was 1%. The blood contained with each concentration of captafol was incubated at 37℃ C for 2 hours under 5% CO_2 gas. The whole blood and plasma were examined for hematological values, erythrocyte membrane damage and biochemical values, respectively.

The results obtained were summarized as follows ;

1. The number of RBC in 1×10^{-2}M group and the concentration of MCHC in 1×10^{-3}M group were significantly ($p<0.05$) decreased and increased from that of control values, respectively. The percentage of Hct was significantly ($p<0.05$) decreased with dose-response.

2. Erythrocyte fragility rate of 1×10^{-3}M and 1×10^{-2}M group were significantly ($p<0.01$) increased from

that control with dose response.

3. Potassium ion level of 1×10^{-2}M was significantly ($p<0.05$) increased from that of control.

4. The concentration of total bilirubin in the 1×10^{-3}M and 1×10^{-2}M groups were significantly increased from that of control. The enzyme level of creatine kinase in 1×10^{-2}M group was significanlty ($p<0.05$) decreased from control value.

Reference

1. Lukens RJ, Sisler HD. Chemical reaction involved in the fungitoxicity of captan. *Dissertation Abstr* 1958 ; 19 : 29~30.
2. Owens RG, Blaak G. Reactions of dichlone and captan with thiols. Contri. *Boyce Thompson Inst* 1960 ; 20 : 475~497.
3. Owens RG. Methabolism of fungicides and related compound. Ann. *N Y Acade Sci* 1969 ; 160 : 141~132.
4. Richmond DV, Somers E. Studies of the fungitoxicity of captan. Ⅳ. Reactions of captan with cell thiols. Ann. *Appli Biol* 1866 ; 57 : 231~240.
5. Siegel MR, Sisler HD. Fate of phtahlimide and trichloromethylthio(SCCl$_3$) moieties of folpet in toxic action on cells of *Saccharomyces pastorianus*. *Phytopathology* 1968 ; 58 : 1123~1128.
6. Liu MK, Fishbein L. Reactions of captan and folpet with thiols. *Experientia* 1967 ; 23(2) : 81~82.
7. Lukens RJ. The fungitoxicity of compounds containing a trichloromethylthio group. *J Agri Food Chem* 1986 ; 14 : 365.
8. Lukens RJ. Hetrocyclic nitrogen compounds, in "fungicides" Torgeson DC. ed. *Academic Press* New York 1969 ; Vol. Ⅱ : 395~423.
9. Lukens RJ. *"Chemistry of Fungicidal Action,"* Springer-Verlag New York 1971 ; 56~72.
10. Carter RJ. Role of sulfhydryl groups in erythrocyte membrane structure. *Biochemistry* 1973 ; 12 : 171~176.
11. Kumer SS, Sikka HC, Saxena J, et al. Membrane damage in human erythrocyte caused by captan and captafol. *Pesticide Biochemistry and Physiology* 1975 ; 5 : 338~347.
12. Gordon HH, Nitowsky HM, Cornblath M. Studies of tocopherol deficiency in infants and children. 1. Hemolysis of erythrocytes in hydrogen peroxide. *Am J Dis Chid* 1955 ; 90 : 669~681
13. Nelson BD. Induction of mitochondrial swelling by the fungicide captan [N-(trichloromethylthio)-4-cyclohexene-1, 2-dicarboximide]. *Biochem Pharmacol* 1971 ; 20(4) : 749~758.
14. Rothstein A, Takeshita M, Knauf PA. Chemical modification of proteins involved in the permeability of the erythrocyte membrane to ions, in "Biomembranes" Kreuzer F. and Sleggers JFG, Eds. Plenum, New York 1971 ; Vol. Ⅲ : 393.
15. Sutherland RM, Stannard NJ, Weed RI. Involvement of sulfhydryl groups in radiation damage to human erythrocyte membrane. Int. *J Radiat Biol* 1967 ; 12(6) : 551~564.
16. Watts DC. Creatine kinase, in Boyer, The enzymes, Academic Press, New York 1963 ; 442.
17. Richterich R, Colombo JP. *Clinical chemistry*, John Wiley & Sons 1981 ; 516~517.