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Toxic Effect of Non-Poisonous Snake Plasma on Various Animal Cells *In Vitro* and *In Vivo**

韓國產 無毒蛇血淸의 各種動物細胞 및 마우스에 對한 毒性

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INTRODUCTION

While observing the proliferation of Japanese encephalitis (JE) virus in snake in 1965, it was found that the plasmas of the Korean common snakes had strong cytotoxic effects on various kinds of animal cells *in vitro* and *in vivo*.

There has been no report that serums of poikilothermic animals show strong toxic effect on various animal cells or animal itself.

It is to be reported that the plasmas from 210 non-poisonous snakes collected in fall of 1965 and 55 snakes collected in early spring of 1966 exerted severe toxic effect on various kinds of red blood cells, tissue culture cells and on mice inoculated intracerebrally.

MATERIALS AND METHODS

Snakes: The snakes caught in Korean fields or mountains of South Korea were non-poisonous Korean common snakes. The plasmas used in this experiment were collected from the following 4 species;

a) Natrix tigrina lateralis Berthold
b) Elaphe rufodorsata Cantor
c) Elaphe schrenckii Strauch
d) Dinodon rufozonatum rufozonatum Cantor

Collection of Blood: Before collecting blood, heparin solution of 1,000 u/ml was applied in drops to the tails of the snakes. By snipping the tail of the snake with sharp knife, the blood was collected in 13 × 100 mm test tube and the plasma was seperated by centrifuging the blood at 1,500 rpm for 10 minutes. Some of the plasma were used immediately after the seperation but the rest otherwise, was kept in −20°C refrigerator prior to use.

Red Blood Cells (RBC): Following 5 kinds of red cells were used.

a) Sheep RBC
b) Human RBC (type A)
c) Chick RBC (1 day old)
d) Rabbit RBC (New Zealand White Rabbit)
e) Mouse RBC (3 to 4 weeks old Swiss Albino Mouse)

To use in examining the hemolytic action of the plasmas on various kinds of red blood cells, 0.25% suspension of the packed red cells, centrifuged at 1,000 rpm for 10 minutes after washing three times with 0.85% saline, was prepared in 0.85% saline solution. Into the test tube of 13 × 100 mm, 0.9 ml of 0.25% red cell suspension and 0.1 ml of the snake plasma was wixed well and the degree of hemolysis was read after 10 minutes at room temperature.

Tissue Culture Cells: Following 4 kinds of cells were cultured in screw cap tubes and 2 oz prescription bottles by the same methods of cultivation[1,2,3,4] as reported.

a) Primary chick embryonic cells

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b) Porcine kidney cells (PS strain)
c) HeLa cells
d) Monkey kidney cells (MS strain)

For testing the cytotoxic effect of the snake plasma on various animal cells *in vitro*, one drop of the plasma was added to 0.9 ml of fluid media with capillary tube and 0.2 ml of the plasma was added directly on the cells grown in 2 oz bottle and adsorbed at 36°C for 120 minutes as in plaque formation with agar overlay.

Swiss Albino Mice: Less than 4 days old mice and 3 to 4 weeks old mice were used.

To suckling mice, 0.01 ml of the plasma was inoculated intracerebrally and, to the adult mice, 0.03 ml was injected intracerebrally and 0.05 ml was injected intraperitoneally. The lethal effect was observed for one week after inoculation.

To destroy the toxic effect of the plasmas,

a) the plasmas were heated at various temperature in water bath for 30 minutes,
b) Na-azide and KIO_3, 1:10,000 final concentration, respectively, were added to the plasmas and kept at the room temperature for ten minutes and
c) ethyl-ether was mixed with the plasma reaching the final concentration 1:5 and kept at 4°C for 30 minutes.

Toxic effect of such treated plasmas on various kind of cells and mice were tested as described.

RESULTS

The Hemolytic Action of Snake Plasma on the Red Blood Cells of Various Animals

As shown in Table 1, plasma of Natrix tigrina lateralis Berthold caused hemolysis of the red blood cells of chick, human, mouse, rabbit and sheep, and the hemolysis occurred generally within 10 minutes. And it was observed that the hemolysis of the chick RBC was mildest. This kind of hemolysis occurred with the plasma even heated at 37°C for 30 min., but the plasma heated at 56°C for 30 min. lost its hemolytic activities completely.

Table 1. Hemolytic action of snake plasma (S-208 Natrix tigrina lateralis) treated at different temperatures to various animal RBC

RBC	Snake plasma		
	Non treated	37°30′	56°30′
Chick	±[1]	±	—
Human	###	###	—
Mouse	###	###	—
Rabbit	###	###	—
Sheep	###	###	—

[1] Read hemolysis after 60′ at 20°C

Table 2 shows the titer of the hemolysis and the fact that Elaphe Schrenckii Strauch plasma cause complete hemolysis of human, mouse and rabbit red blood cells even in the plasma-dilution of 1:100.

Almost same degree of hemolytic titers was also observed in pooled snake plasma.

It was also observed that Elaphe schrenckii Strauch plasma can cause more severe hemolysis on chick RBC than that of Natrix tigrina lateralis Berthold plasma.

Table 2. Hemolytic action of snake plasma (S-207 Elaphe schrenckii Strauch) to various animal RBC

RBC	Dilution of snake plasma						
	1:50	1:100	1:200	1:400	1:800	1:1,600	Control
Chick	###[1]	±	—	—	—	—	—
Human	###	###	###	+	±	—	—
Mouse	###	###	++	+	—	—	—
Rabbit	###	###	###	±	—	—	—
Sheep	###	++	—	—	—	—	—

[1] Read hemolysis after 60′ at 20°C.

The Influence of Temperature and Enzyme Blockers on The Toxic Action of Snake Plasma

In Table 3 the following problems were tested. a) At what temperature is the toxicity of the plasma inactivated? b) Is this toxic effect due to the enzymatic activities present in the snake plasma?

The hemolytic action of E. Schrenckii Strauch plasma could not be inactivated by heating at 42°C for 30 minutes but heating at 47°C for 30 minutes could prevent the hemolysis of sheep and mouse red blood cells. Complete destruction of the hemolytic activity could be obtained by heating the plasma at 51°C for 30 minutes.

For the second question, 0.03 ml of 0.1% Na-azide and KIO_3 solutions were mixed with 0.27 ml of the plasmas respectively and the toxicity was tested after keeping the mixtures at room temperature for 10 minutes. The enzyme blockers so far that were employed could not inhibit the hemolytic ability of the snake plasma.

The result with the plasma of Natrix tigrina lateralis Berthold was almost same with that of E. schrenckii Strauch.

Table 3. Hemolytic action of snake plasma (S-207 E. schrenckii) treated with enzyme blockers and at different temperatures to various animal RBC

Snake plasma treated	RBC				
	Chick	Human	Mouse	Rabbit	Sheep
Sod. azide[1]	###	###	###	###	###
KIO_3[1]	###	###	###	###	###
42°C 30′	##	###	###	###	###
47°C 30′	±	###	—	##	—
51°C 30′	—	—	—	—	—
56°C 30′	—	—	—	—	—
Control	—	—	—	—	—

[1] 1 : 10,000 final dilution

Cytotoxic Effect of Snake Plasma on Animal Cells In Vitro

The purpose of the experiment shown in Table 4 is to examine the cytotoxic effect of the snake plasma on various kinds of cultured cells. The snake plasma had strong cytotoxic action on primary chick embryo cells, porcine kidney cells, HeLa cells and monkey kidney epithelial cells but the plasma heated at 47°C for 30 minutes lost its cytotoxic effect.

The cytotoxic action of the plasma appeared so rapid that the addition of the plasma to cultured cells induced immediate cell degeneration.

Lethal Effect of Snake Plasma on Swiss Albino Mouse

Table 5 shows the result of the lethal effect of the plasma on Swiss Albino mice. Intracerebral inoculation of the plasma either to suckling mice or adult mice showed severe lethal action and most of the mice were killed within a few minutes after inoculation. The mice appears to be subjected to fatal hit at once as shown by severe convulsion immediately after the inoculation, thus, even the survived mice right after inoculation could not live longer than 24 hours. It was not effective to minimize the lethal effect of the plasma by mixing the plasma with equal amount of normal mouse brain suspension or horse, swine and rabbit serums respectively. However, intra-

Table 4. Cytotoxic effect of pooled snake plasma to animal cells grown *in vitro*

Snake plasma	Adsorption of plasma at 36°C 90′	Primary chick embryo cells	Porcine kidney cells	HeLa cells	Monkey kidney cells
Non-treated plasma	0.2 ml/bottle	ACD[1]	ACD	ACD	ACD
42°C 30′ plasma	〃	ACD	ACD	ACD	ACD
47°C 30′ plasma	〃	Healthy	Healthy	Healthy	Healthy
51°C 30′ plasma	〃	Healthy	Healthy	Healthy	Healthy
56°C 30′ plasma	〃	Healthy	Healthy	Healthy	Healthy

[1] All cell are dead.

Table 5. Lethal effect of non-poisonous pooled snake plasma to Swiss-albino mice

Snake plasma	Age of mouse	Route and dose	No. of death / No. of Inoculated
Non-treated	3~4 wks	I.C. 0.03 ml	46/53
Non-treated 10^{-1} dil.	〃 〃	〃 〃	10/15
10^{-0} pl+10^{-3} NMB[1] 37°60′	〃 〃	〃 〃	5/5
10^{-0} Pl. 56°30′	〃 〃	〃 〃	0/9
Non-treated plasma	1~5 days	I.C. 0.01 ml	10/10
10^{-0} Pl. 56°30′	〃 〃	〃 〃	0/7
Non-treated plasma	3 wks	I.P.&S.C. 0.5 ml	0/4
Non-treated plasma	6 days	I.P. 0.05 ml	0/5
Non-treated plasma + Horse serum 36° 30′	4 wks	I.C. 0.03 ml	4/5
Non-treated plasma + Swine serum 36° 30′	〃 〃	I.C. 0.03 ml	5/5
Non-treated plasma + Rabbit serum 36° 30′	〃 〃	I.C. 0.03 ml	4/5
Plasma treated with 1:4 dil. of ethyl-ether	3 wks	I.C. 0.03 ml	0/5

[1] 10% normal mouse brain suspension

peritoneal or subcutaneous injection of the plasma has shown no lethal effect.

Toxic Effects of Normal Snake Tissues on Mice

Another experiment to learn the toxic effect of snake tissues other than plasma has been understaken. As shown in Table 6, suspensions of muscle, liver and spleen have no any toxic effect so far. Attempts were also made to demonstrate the enzymatic action of the snake plasma. Urease and lecithinase activities were tested but according to the present results shown in the Table 7, there are no such activities in the snake plasma used and nor exotoxin-like activities were detected.

Table 6. Toxic effect of normal snake tissues to mouse[1]

Materials	Route & dose of inoculation	No. mice dead / No. mice inoculated
Snake Plasma		
10^{-0}	I.C. 0.03 ml	10/10
10^{-1}	I.C. 0.03 ml	2/5
Snake muscle		
10^{-1}	I.C. 0.03 ml	0/4
Snake liver & spleen		
10^{-1}	I.C. 0.03 ml	0/20

[1] 3-5 weeks old Swiss albino mice

Table 7. Enzymatic action of snake plasma

Snake plasma	Urease test	Lecithinase test
S-207	—	—
S-224	—	—

Experiment for Removing Toxicity of The Snake Plasma

In order to seperate any toxic fraction of snake plasma, euglobulin and pseudoglobulin were separated by ammonium sulfate method, but both fractions showed the same toxic effect as much as control. Besides the experiment, the precipitates obtained after dialysis have also been tested but the toxic effect remained as before.

To separate ether soluble fraction from the plasma, four parts of snake plasma and 1 part of ethyl ether were mixed and kept at 4°C for 30 minutes before centrifugation. After removal of ether by centrifugation, the resulting plasma was proved to be enirely free of toxic action as shown in Table 5.

DISCUSSION

In tissue culture process, the serums of various animals contained in the growth media, generally show growth-promoting effect, but certain lots of the serum show rather cytotoxic effect on the cells. Such phenomenon usually can be found with observation of the cell-growth after 2 or 3

days of incubation.

While studying on the isolation of virus from the snake blood collected in the fields and the proliferation of virus in the virus inoculated snakes, it was observed that snake plasma has cytotoxic effect on various kinds of cells and lethal effect to mice when inoculated intracerebrally.

There has been no report on such strong cytotoxic effect of non-poisonous common snake plasma as shown in the results. And it was also found that the plasma has direct hemolytic ability on RBC of various animals. As shown in the results, red blood cells of human, rabbit, sheep and mouse were hemolized by the plasma and the hemolysis was generally observed within 5~10 minutes after mixing the RBC and the plasma, but the plasma heated at 56°C for 30 min. did not show any hemolytic effect. On the contrary cytotoxic effect of serums of warm blooded animals to tissue culture cells are not destroyed by heating at 56°C 30 min.

Thus, a heat-labile substance of the plasma is supposed to be related with the hemolysis.

Table 2 shows that the plasma diluted above 1:100 induced the hemolysis. This kind of hemolysis was observed in the plasmas of 4 species of the snakes employed in this experiment and the pooled plasma showed also the same degree of hemolysis.

It was studied whether this cytotoxic effect was caused by any enzyme contained in the plasma, but the employed enzyme blockers such as Na-azide or KIO_3 could not prevent the hemolytic effect, thus none of enzyme is seen to be concerned with the hemolytic effect.

According to the study on the attitude of the plasma to various temperatures, heating the plasma at 47°C for 30 min. could induce only incomplete destruction of the hemolytic ability but at 51°C for 30 min. completely destroyed the ability. In other words, for the complete destruction of the hemolytic ability, it is not necessary for the plasma to be heated at 56°C for 30 min. and it was also known that a heat-labile substance that can be destroyed at 51°C for 30 min. is related with the hemolysis.

Primary chick embryo cells and porcine kidney cells are generally being used for JE virus isolation and the antibody-analysis.

For the purpose of studing plaque-formation of the virus, 0.2 ml of the snake plasma was directly adsorbed on the cells for 120 minutes at 36°C. By such procedures the four kinds of cells we have in the laboratory showed immediated destruction. The plasma heated at 47°C for 30 min. did not show any effect on these cells and same results were observed in HeLa cells and monkey kidney cells.

In porcine kidney cell culture in screw-cap tubes, the addition of a drop of the plasma with a capillary tube to 0.9 ml of the fluid media induced immediate death of the cells, and even 0.1 ml of the plasma diluted at 1:10 caused rapid cell destruction and increase in pH of the media. And the longer we kept the plasma at −20°C the weaker the cytotoxic effect became.

To study in what tissues of the snakes the toxic substance is contained, the various tissues including brain tissue of snakes were examined. The toxic effect was proved only in the plasma but muscle, liver, spleen or brain suspensions did not show any cytotoxic effect.

Pigs are not sensitive to snake-poison and even the bite of poisonous snakes may not cause any remarkable damage to pigs. Therefore, it was supposed that certain minimizing or neutralizing substances might be contained in the plasma of pig. To observe such effects on snake plasma it was studied whether any neutralizing substance is contained in various animal serums. The snake plasma was mixed with equal amount of the serums of pig, horse, and rabbit respectively. This plasma and serum mixture was injected into mice intracerebrally after keeping at 36°C for 30 minutes and it was shown that the mixtures were as much toxic as the plasma alone. In adult mice, the

intracerebral inoculation of the snake plasma caused severe convulsion and immediate death as in suckling mice. But intraperitoneal or subcutaneous injection of the plasma did not show any toxic effect, even in case of the injection of relatively large amount of the plasma.

These findings show that this toxic substance is thought to have specific affinity with brain tissue.

The toxic effect could not be erradicated by dialysis of the plasma or precipitation of euglobulin with ammonium sulfate.

However, the toxic effect can be completely removed by mixing the plasma with ethyl ether. Since ether is a chemical solvent of lipid, the toxic substance seems to be related with ether-soluble lipid.

To explore whether the toxic substance is the exotoxin-like substance or enzyme in nature, urease and lecithinase test were carried out but both results were negative.

More study is required for complete understanding of the chemical nature of the toxic substance in snake plasma.

SUMMARY

According to the test of the plasma from 265 non-poisonous common snakes of Korea, the plasma showed strong toxic effect on various animal cells, and IC inoculation of the plasma into mice showed lethal effect.

1) The snake plasma induced strong hemolysis of chick, human, mouse, rabbit and sheep RBC up to dilution of 1:100.
2) The hemolytic ability of the plasma was completely destroyed by heating at 51°C for 30 minutes.

   The cytotoxic effect on various tissue culture cells was completely destroyed by heating at 47°C for 30 minutes.
3) This toxic substance was seen neither an enzyme nor an exotoxin-like substance. The plasma treated with ethyl ether lost the toxicity completely.
4) Intracerebral inoculation of the snake plasma into mice showed immediate lethal action.

=國文抄錄=

韓國產無毒蛇血淸의 各種動物細胞 및 마우스에 對한 毒性

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溫血動物이나 冷血動物의 血淸이 强한 細胞파괴력과 쥐 腦內에 注射時 致死作用을 나타낸다는 報告는 아직 없다.

여기에 報告하는 것은 이같은 作用을 처음 觀察한 것으로 韓國에서 採集한 265마리의 無毒蛇의 血淸을 檢査한 結果 이들 血淸은 各種 動物細胞에 强한 毒性을 나타내었으며 또 쥐에 對하여 致死作用을 나타내었다.

(1) 뱀의 血淸은 닭, 사람, 쥐, 羊의 赤血球에 强한 溶血作用을 나타냈으며, 또 100倍로 희석된 血淸으로 實驗해도 같은 效果가 있었다.

(2) 뱀의 血淸의 溶血作用은 51°C에서 30分間 加熱하면 完全히 파괴되었다.

培養한 各種 動物細胞에 對한 파괴력은 47°C에서 30分間 加熱하면 完全히 消失되었다.

(3) 뱀 血淸中의 毒性物質은 酵素가 아니었으며 또 細菌의 體外毒素(Exotoxin)같은 物質도 아닌 것 같다.

Ethyl-ether로 抽出하면 이 血淸은 그 毒性을 完全히 喪失했다.

(4) 뱀 血淸을 쥐 腦內에 注射하면 즉시 致死作用을 나타냈다.

REFERENCES

1) J.S. Porterfield: *Bull Wld. Hlth. Org., 22;373, 1960.*
2) H. Kato and Y.K. Inoue: *Virology, 18;500, 1962.*
3) Y.M. Song., H.W. Lee and C.Y. Park: *J. Korean Medical Association, 7;357, 1964.*
4) J.L. Melnick: *Ann. New York Acad. Sc., 61; 754, 1955.*