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The effects of gamma irradiation on the survival and development of *Clonorchis sinensis* metacercariae†

Soon-Hyung Lee, Yang-Hee Park, Woon-Mok Sohn*,
Sung-Tae Hong and Jong-Yil Chai

Institute of Endemic Diseases and Department of Parasitology, College of Medicine, Seoul National University, Seoul 110-460, and Department of Parasitology, College of Medicine, Inje University, Pusan 614-112, Korea*

Abstract: The effects of gamma irradiation on the survival and development of *C. sinensis* metacercariae were studied to evaluate the feasibility of irradiation as a control measure for clonorchiasis. *Pseudorasbora parva* were collected at an endemic river of clonorchiasis and were used for irradiation of the fluke in three schemes. The first (Scheme 1) was irradiation of the isolated metacercariae from the fish followed by infection to experimental rats. The second (Scheme 2) was irradiation of the fish, and then the metacercariae were isolated and infected to rats. The third (Scheme 3) was irradiation on the rat livers after infection with normal metacercariae. Irradiation doses varied from 5 to 100 Gy for Schemes 1 and 2, and 10 to 25 Gy for Scheme 3. The rats were sacrificed 2 to 6 weeks after infection.

In Scheme 1, the metacercariae irradiated at 50 Gy failed to survive in the rats after 2 or 6 weeks. However, 1 to 44% of the metacercariae irradiated at 5~30 Gy survived. The estimated LD_{50} of Scheme 1 was 16.5 Gy. The flukes irradiated in Scheme 2 survived better than those in Scheme 1. The average worm recovery rate in 50 Gy was 28%(7~39% individually). Increasing the dose up to 100 Gy brought a remarkably low survival rate of an average 1%(0~3% individually). The LD_{50} of Scheme 2 was 47.5 Gy. Worm recovery rates in the 10 Gy group of Scheme 3 were 21~39%, and those in the 25 Gy group were 2% and 34%. Although the metacercariae were irradiated, all of the recovered worms were morphologically normal. Only the worms irradiated with 10 Gy or 25 Gy after 9 days from infection in Scheme 3 showed underdeveloped testes and seminal receptacle.

The present results suggest that irradiation of the fish by 100 Gy could be adopted as a control measure for clonorchiasis.

Key words: *Clonorchis sinensis*, gamma irradiation, survival, development

INTRODUCTION

The Chinese liver fluke, *Clonorchis sinensis*, has been one of the most important human parasitic helminths in Korea. In recent years its prevalence in endemic areas has been decreasing due to the strenuous efforts in health education and wide application of an effective drug, praziquantel(Rim, 1986). However, it is still highly prevalent to draw an attention from the public health point of view. Human

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infection is contracted by eating raw fresh water fishes which harbour the metacercariae of *C. sinensis*. Therefore, it might be controlled if the metacercariae in the fish were killed selectively.

Ionizing radiation was found to have various effects on helminth parasites. Only a small proportion of *Schistosoma mansoni* cercariae which were irradiated at 23 Gy could grow to adults, and the majority of the females were sterile(Bickle *et al.*, 1979). The migration activity of schistosomula was also weakened by irradiation(Bickle *et al.*, 1979). Such effects of radiation were also reported in other helminths such as *Fasciola hepatica*(Dawes, 1963), *Toxocara canis*(Barriga and Myser, 1987), *Angiostrongylus cantonensis* (Ishii *et al.*, 1987) and *Hymenolepis microstoma*(Djeon, 1965). Most of the irradiated helminths were either significantly attenuated or led to death depending on the doses of radiation. Thus, irradiation at the infective stage is being applied to vaccination against schistosomiasis, for example (Gordon and McLaren, 1987). Another application of irradiation is as a control measure against parasitic infections transmitted by food. Control of trichinosis or other food-borne helminthiasis by irradiation of meat or fish is a promising subject for investigation.

The purpose of the present study is to observe the effects of gamma irradiation on the survival and development of *C. sinensis* and ultimately to evaluate the feasibility of irradiation as a control measure for clonorchiasis.

MATERIALS AND METHODS

1. Collection of *C. sinensis* metacercariae

Hundreds of *Pseudorasbora parva*, a species of fresh water fish in 5~8 cm length, were collected at the Nakdong River near Pusan where clonorchiasis is endemic. From the digested material of the fish, the metacercariae were isolated under stereomicroscopy. They were washed and stored in cold physiological saline until ready for use.

2. Experimental schemes

Irradiation of metacercariae(Scheme 1) : 1,000 metacercariae were dispensed to each petri dish(8 cm in diameter) containing 20 ml saline. The dishes were put on the rounding plate of MK 1-68 Cs^{137} gamma-irradiator(JL Sheperd and Associates Co.) and irradiated at the rate 3 Gy/0.79 min at 40 cm distance. The radiation dose was adjusted from 5 Gy (1Gy= 100 rad) to 50 Gy, changing the exposure time.

Irradiation of fish (Scheme 2) : The fish, *Pseudorasbora parva*, were also irradiated after their death at doses from 10 to 100 Gy, half on one side and half on the other. After irradiation the metacercariae were isolated from the digested material of the fish and used for infection of the rats.

Irradiation of rats (Scheme 3) : The rats infected with 100 unirradiated metacercariae of *C. sinensis* were irradiated on their livers(including the lower chest and upper abdomen) on the second, fifth or ninth day after infection. With their chest upward on a wooden plate, they were exposed to gamma irradiation while other parts of the body were lead-shielded (5 cm thickness). The radiation source was Co^{60} (Picker C-9), and the distance from the source to the skin was 70 cm. Field size was 10×10 cm, and the dose rate was 161.8 Gy/min.

3. Experimental infection of the rats

Albino rats of the Sprague-Dawley strain, supplied by the Laboratory Animal Center, Seoul National University, were orally infected with either irradiated or unirradiated metacercariae, according to the schemes, through a gavage needle. Each rat was given 100 metacercariae.

4. Recovery and morphological observation of *C. sinensis*

The rats infected with irradiated metacercariae (Scheme 1 or 2), or the rats irradiated on their livers at the early stage of infection (Scheme 3), were sacrificed by spinal shock 2 or 6 weeks after the infection. Their livers

were removed and chopped for recovery of the worms. The worms were collected by groups, fixed and stained with Semichon's acetocarmine, followed by morphological observation under light microscopy.

RESULTS

1. Morphology of the irradiated metacercariae

The metacercariae which were exposed to gamma irradiation showed no abnormality in their morphology and activity. They showed active movements within their cysts as well as after excystation.

2. Recovery of irradiated *C. sinensis* from rats

A) Scheme 1 (Irradiation of metacercariae) : In the control rats infected with unirradiated metacercariae, about half of the infected *C. sinensis* were recovered at 2 weeks after the infection (Table 1). Compared with this, no worm was recovered from the rats infected with the metacercariae irradiated at 50 Gy. The recovery rate of the worms, however, increased as the irradiation dose decreased, from 1% in the 30 Gy group to 44% in 10 Gy group (Table 1 & Fig. 1). Similar results were obtained in the rats sacrificed six weeks after infection (Table 1 & Fig. 1). Compared with a 63% recovery rate in the control group, no worms were found in the 50 Gy irradiation group (Table 1). The rate, however, increased to 8% in the 30 Gy group and to 44% in the 10 Gy group. The LD_{50} by this irradiation scheme was about 16.5 Gy (Fig. 2).

B) Scheme 2 (Irradiation of fish) : In general, more worms were recovered by this irradiation scheme(Table 2 & Fig. 1) than by that of Scheme 1. The recovery rates were 28 ~80% in the 10~50 Gy groups. However, only 1% of the worms were recovered in the 100 Gy group. The LD_{50} by this scheme was calculated to be 47.5 Gy (Fig. 2).

C) Scheme 3 (irradiation of rats) : Eleven of 12 rats which were irradiated at 10 Gy on the second, fifth or ninth day after infection showed a worm recovery rate ranging from

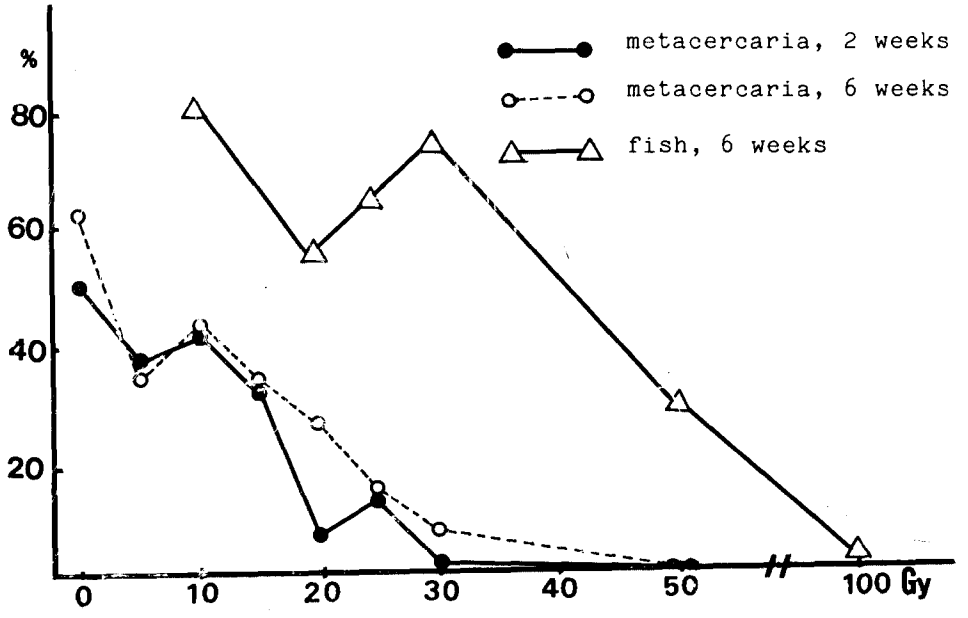

Fig. 1. Recovery rate(%) of *C. sinensis* irradiated on metacercariae or fish, 2 or 6 weeks after infection by gamma-ray dose.

Table 1. Recovery of *C. sinensis* from rats infected with irradiated metacercariae (Scheme 1)

Irradiation dose (Gy)	2 weeks			6 weeks		
	No. of rats*	Worm recovery rate(%)		No. of rats*	Worm recovery rate(%)	
		Mean	Range		Mean	Range
0	5	50	35～65	5	63	31～78
5	4	36	30～43	6	35	20～57
10	8	44	32～54	7	44	19～72
15	9	31	11～52	5	37	31～43
20	7	6	3～10	5	26	18～36
25	8	12	3～17	4	14	5～21
30	7	1	0～ 3	5	8	4～11
50	5	0	0	5	0	0

* Each rat was infected with 100 metacercariae.

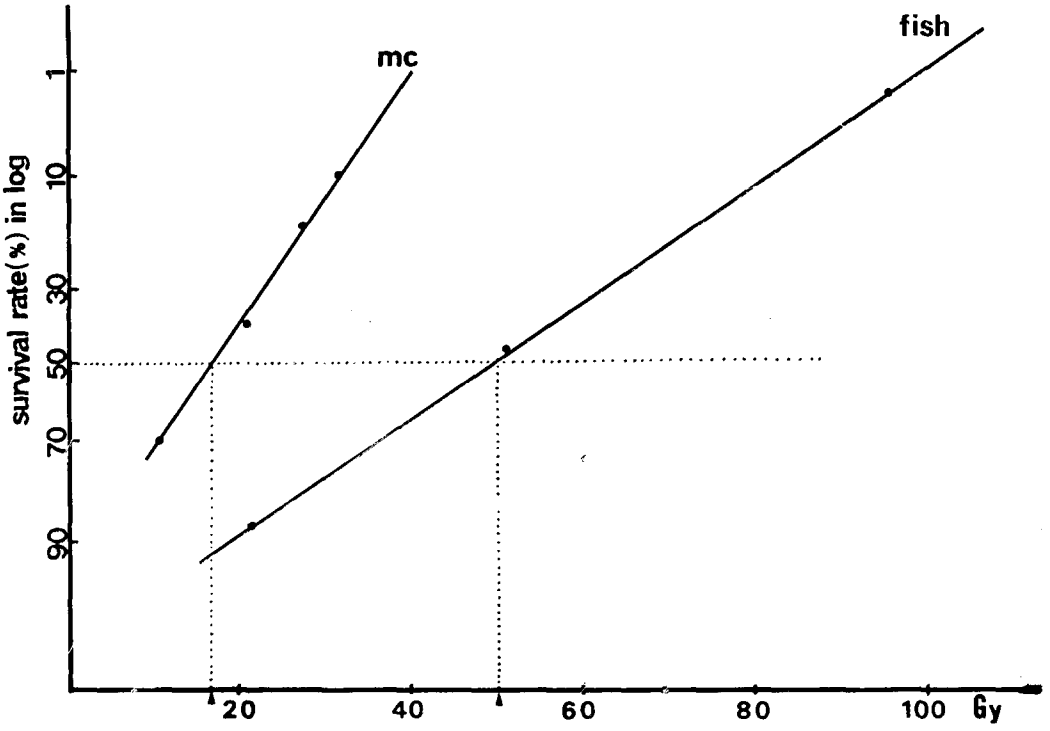

Fig. 2. Survival rate(%) of *C. sinensis* in logarithmic scale by gamma-irradiation dose; irradiation on the metacercariae(mc) or on the fish(fish), arrow heads denote the LD_{50}.

Fig. 1. *C. sinensis* of the control group, 6 weeks old, ×18. (→)

Fig. 2. *C. sinensis* from Scheme 3, 10 Gy irradiated on the 9th day after infection, ×18.

Fig. 3. Magnified posterior part of the worm of Fig. 2. The ovary looks well-developed but the testes are poorly-developed and the seminal receptacle is empty, ×40.

Fig. 4. *C. sinensis* of 30 Gy group irradiated on the metacercariae, ×18. It is morphologically normal.

Fig. 5. *C. sinensis*, irradiated with 25 Gy on the 9th day after infection, ×18. The ovary, uterus, vitellaria and others are morphologically normal except for the testes and seminal receptacle.

Fig. 6. Magnified posterior end of Fig. 5, with underdeveloped testes and empty seminal receptacle. (Abbreviations; O-ovary, S-seminal receptacle, T-testes, U-uterus filled with eggs)


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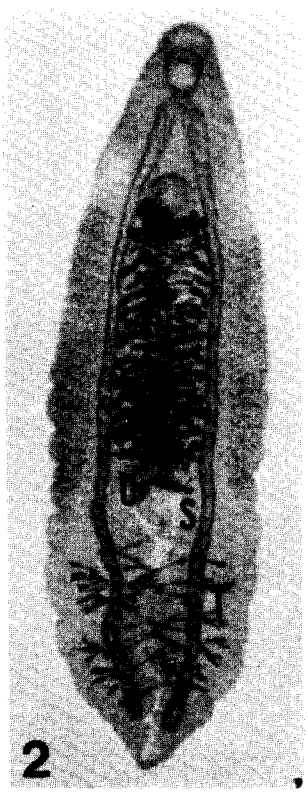
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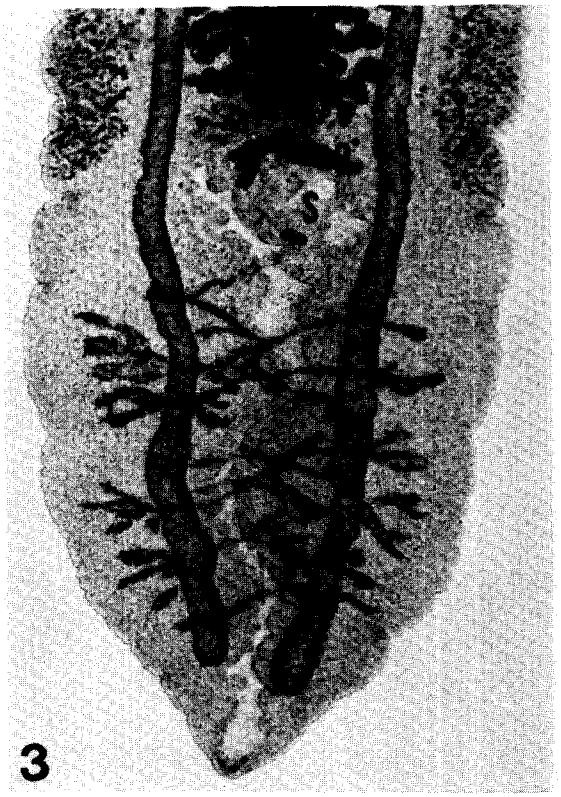
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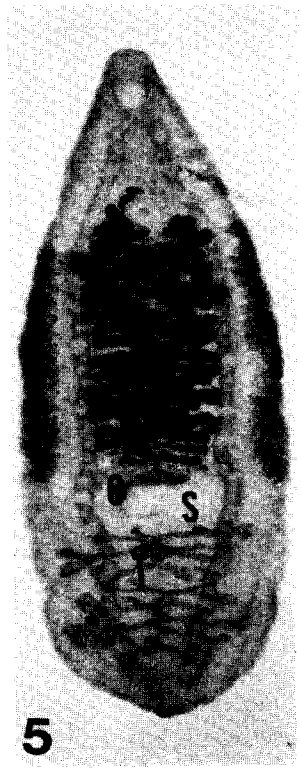
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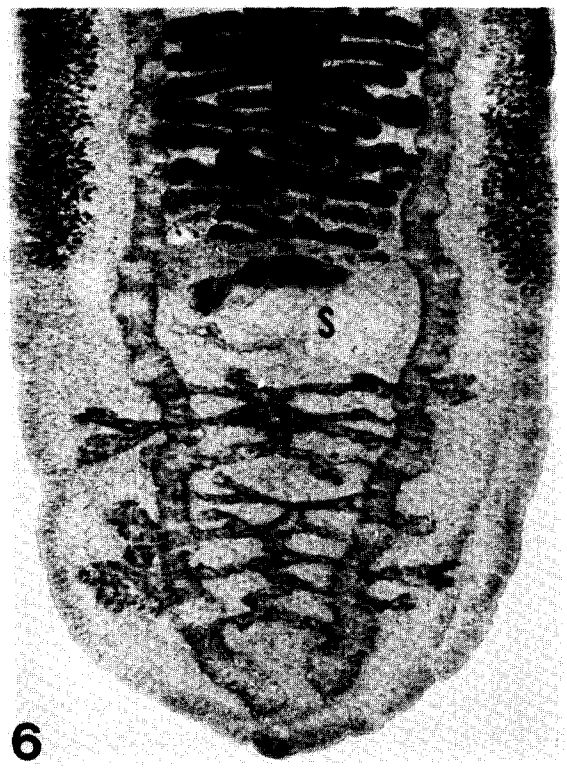
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Table 2. Recovery of *C. sinensis* from rats 6 weeks after infection with the metacercariae irradiated in the fish (Scheme 2)

Irradiation dose (Gy)	No. of rats	Total No. of metacer. infected	Total No. of worms recovered	Worm recovery rate(%)	
				Mean	Range
10	5	500	399	80	71~85
20	4	400	211	55	27~74
25	5	500	321	64	50~79
30	5	500	364	73	55~82
50	6	600	169	28	7~39
100	7	700	8	1	0~ 3

Table 3. Recovery of *C. sinensis* at 6 weeks after infection from rats which were irradiated in early stages of infection (Scheme 3)

Irradiation dose (Gy)	Day of irradiation after infection	No. of rats irradiated	No. of rats survived	Total No. of worms recovered	Worm recovery rate(%)	
					Mean	Range
10	2	4	3	116	39	31~51
	5	4	4	82	21	0~46
	9	4	4	96	24	0~34
25	2	4	1	2	2	2
	5	4	0	—	—	—
	9	4	1	34	34	34

21% to 39% at 6 weeks after infection(Table 3). One rat was dead after irradiation. On the other hand, in the 25 Gy group, 10 of the 12 irradiated rats died within two weeks after irradiation, and only two survived until 6 weeks after infection, revealing a worm recovery rate of 2% or 34%.

3. Morphology of *C. sinensis* after irradiation

The worms which were recovered at 6 weeks after infection were compared by groups. Both immature and mature worms were recovered from the control as well as from the irradiated rats. However, little difference in the morphology of the worms was found between the groups. In all of the groups, most of the surviving worms, except a few, were fully grown and matured. The worms which were irradiated after 9 days from infection in rats at 10 Gy or 25 Gy (Scheme 3) showed underdevelopment of the testes and seminal receptacle. The worms which were irradiated on the second or fifth day after infection at 10 or 25 Gy doses in Scheme 3, however, were morphologically normal(Figs. on the Plate).

DISCUSSION

The effects of ionizing radiation on parasitic helminths are growth retardation, vacuolization of the interstitium, elevation of the tegument, malformation or underdevelopment of the reproductive organs, failure of reproduction, and/or weakened pathogenicity in the host (Hughes, 1972; Villella and Weinbren, 1965). In addition, inhibition of migration is known for the worms that should migrate in their host. Helminths such as *Schistosoma mansoni* (Bickle, 1979), *Fasciola hepatica* (Dawes, 1963), *Toxocara canis* (Barriga and Myser, 1987) or *Paragonimus ohirai* (Ikeda and Tani, 1984) were hindered in their migration, with an increased death rate at the migration sites or with prolonged time of migration.

The most prominent effect of gamma irradiation on *C. sinensis* metacercariae was the reduction of their survival rate in the rat host. The survival rate of the irradiated metacer-

cariae in Scheme 1 decreased as the dose of radiation increased. No worm was recovered after the 50 Gy irradiation by Scheme 1. The number of surviving worms was not different by the duration of infection, 2 weeks and 6 weeks. At present, it is uncertain when the irradiated metacercariae were dead. Immediately after exposure to the radiation they looked completely normal, but they were found dead at their early developmental stages within 2 weeks of infection in the rats.

More worms survived when the infected fish were irradiated (Scheme 2). An average of 73% (55~82%) of the infected worms were recovered from the rats in the 30 Gy group, and as much as 28% (7~39%) were recovered in the 50 Gy group. In the 100 Gy irradiation group, however, only 1% (0~3%) of the infected flukes were recovered. At any rate, the metacercariae of *C. sinensis*, which were irradiated when they were encysted in the flesh of fish, were less susceptible to irradiation than those irradiated after isolation from the fish. As shown in Fig. 1, the LD_{50} was 16.5 Gy in Scheme 1 and 47.5 Gy in Scheme 2.

Such a difference in radiation susceptibility of *C. sinensis* metacercariae between the two schemes may be caused by the differences of the milieu around the metacercariae. Gamma rays penetrate deep into the animal tissue with little loss. Hence, there should have been no difference in the doses that arrived at the metacercariae between Schemes 1 and 2. In Scheme 1, the metacercariae were stored in 20 ml 4°C physiological saline after isolation from the fish and were irradiated in a small petri dish, while the infected fish were irradiated in Scheme 2. Therefore, only the difference was in the surrounding media, such as saline or the flesh of the dead fish. Radiation is known to destroy DNA structures directly or by forming hydroxy radicals in the tissue, so that there may be three times more cell destruction in an oxygenated state than in an anoxic one(Hall, 1978). It is suggested that the flesh of the dead fish should have been anoxic, therefore, there was less destruction of cells, and the irradiated metacercariae in the flesh survived more.

Clonorchis in this study is quite sensitive to radiation compared with other helminths. The LD_{50} was 16.5 Gy and none was alive at 50 Gy. One-percent of the *Schistosoma* adults were recovered after irradiation at 80 Gy (Smithers, 1962; Erickson, 1965), and migrating schistosomules became undetectable in the lungs only after irradiation at 200 Gy(Bickle *et al.*, 1979). *P. ohirai* was also sensitive to irradiation and were dead in the liver after exposure to 20 Gy (Ikeda and Tani, 1984). Contrary to this, the larvae of *Toxocara canis* survived even after receiving 1,500 Gy radiation(Barriga and Myser, 1987). Such a difference is seen in various species of parasites but may be seen as well in different stages by which the frequency of cell division is determined, since it is well-known that the more cell division the more sensitive to irradiation.

It is an interesting finding that the *C. sinensis* irradiated on the ninth post-infection day revealed underdeveloped testes and empty seminal receptacle but the uterus was full of numerous eggs. The worms which were irradiated when they were metacercariae or earlier than 5 days of infection, however, were morphologically normal after 6 weeks of infection in rats in spite of high doses of irradiation. Therefore, it is speculated that oogenesis was still possible while spermatogenesis was inhibited by 10 Gy irradiation on the ninth day after infection. Such an effect of radiation depends on the degree of cell division during organogenesis at a certain stage, and thus the actively developing worms are more critical than the metacercariae by irradiation although the survival of worms correlates with the irradiation dose. The present finding suggests that the testes of *C. sinensis* actively develop at nine days after infection. It was reported that only a small proportion of irradiated *Schistosoma mansoni* were found living, and both spermatogenesis and oogenesis were suppressed by 23 Gy radiation (Bickle *et al.*, 1979). In the worms

with separate sexes, males were found more sensitive to radiation than females as observed in *Angiostrongylus cantonensis*(Ishii *et al.*, 1987) and also in some arthropods such as *Amblyomma* (Oliver and Stanley, 1987) or *Dermanyssus* (Entrekin *et al.*, 1987).

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=국문초록=

감마선 照射가 흰쥐에서의 肝吸蟲 생존 및 발육에 미치는 영향

서울대학교 의과대학 풍토병연구소 및 기생충학교실, 인제대학교 의과대학 기생충학교실*
이순형 • 박양희 • 손운목* • 홍성태 • 채종일

우리 나라에서 가장 중요한 인체 기생충의 하나인 간흡충(*Clonorchis sinensis*)의 감마선에 대한 영향과 그 관리책으로 감마선 照射의 효용성을 관찰하고자 이 연구를 시행하였다.

김해산 참붕어를 수집하여 이를 소화시켜 피낭유충을 분리하고, 분리된 피낭유충을 照射한 실험군(1군), 죽은 참붕어를 照射한 후 피낭유충을 분리한 군(2군), 피낭유충을 흰쥐에 감염시키고 감염 초기에 간을 照射한 군(3군)으로 나누어 실험을 하였다. 照射된 피낭유충은(1,2군) 흰쥐에 실험감염시키고 3군의 실험동물과 함께 감염 2주 및 6주 후에 도살하여 감염된 충체를 회수하였다. 감마선 照射量은 5 Gy(1 Gy=100 rad)부터 100 Gy의 범위에 있었다.

그 결과를 요약하면 아래와 같다.

1. 분리한 피낭유충을 照射한 후 감염시킨 1군에서는 감염 2주에 평균 충체 회수율이 5, 10, 15 Gy 照射量에 따라 각각 36.5%, 44.1%, 30.7%이었고, 20 Gy, 25 Gy, 30 Gy 照射量에서는 평균 5.7%, 11.8%, 1.0%가 회수되었다. 照射量 50 Gy 군에서는 한 마리도 검출되지 않았다. 감염 후 6주의 충체 회수율도 대조군에서 62.6%이고, 照射量이 증가함에 따라 감소하였고 30 Gy 군에서 7.8%, 50 Gy군에서 0%이었다. LD_{50}는 16.5 Gy이었다.

2. 참붕어를 직접 照射한 2군의 감염 6주 후 충체 회수율은 10 Gy에서 79.8%이었으나, 照射量이 늘어남에 따라 20 Gy에 55.3%, 25 Gy에 64.0%, 30 Gy에 72.8%, 50 Gy에 28.2%, 100 Gy에 1.1%로 감소하였다. LD_{50}은 47.5 Gy로 계산되었다. 이러한 소견은 간흡충의 피낭유충이 죽은 생선의 근육내에 있을 경우에, 분리하여 식염수에 넣은 채 노출시킬 때보다 감마선 照射에 훨씬 저항성이 있음을 보여준다.

3. 제 3 군의 감염 초기에 照射하고 감염 후 6주에 관찰한 경우 10 Gy 照射에서 감염 2일 후 照射에 의해 39%(31~51%), 5일과 9일 후에 각각 21%(0~46%) 및 24%(0~34%)가 회수되었다. 照射量을 25 Gy로 증가시킨 후에는 대부분의 흰쥐가 충체 회수 전에 사망하여, 감염 2일과 9일 후에 照射한 군에서 각각 한 마리 씩에서만 2마리 및 34마리의 충체를 얻었다. 이 결과는 같은 照射量에 노출된 피낭유충의 생존률과 같은 수준이었다.

4. 감마선에 노출된 피낭유충이나 흰쥐에 실험감염 후 생존한 충체를 관찰한 결과 형태학적인 이상은 관찰되지 않았다. 다만 흰쥐에 감염시키고 9일 후에 10 Gy 및 25 Gy를 照射한 충체에서는 고환과 저정낭의 발육이 매우 부진하였다.

이상의 결과로 미루어 보면, 간흡충은 감마선에 비교적 예민하며 100 Gy의 照射量을 죽은 어류에 조사하면 간흡충 피낭유충의 감염력이 거의 없어지는 것을 확인하였다. 죽은 魚體 안에 있는 피낭유충이, 이미 분리되어 식염수에 담겨진 것이나 동물 감염 후 2~9일에 감마선 照射에 노출된 충체보다 더 많이 살아 남았는데, 이는 죽은 魚體가 무산소 환경이기 때문에 hydroxy radical(OH-)이 적게 생기기 때문으로 생각된다. 또한 감마선에 노출된 충체의 대부분은 외형적으로 온전하고 산란도 정상으로 하였으나, 감염 9일 후에 照射된 것에서는 고환의 발육 부진과 정자 생성의 저해가 관찰되었다. 감마선 照射를 실제 간흡충 관리에 적용하기 위해서는 일상생활에서 이 방법의 응용 가능성에 대한 연구가 더 필요하다고 생각된다.