

The Wormicidal Substances of Fresh Water Fishes on *Clonorchis sinensis*

VII. The Effect of Linoleic Acid and Ethyl Linoleate on Parasite Viability

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Abstract: In an attempt to analyze the clonorchicidal activity of linoleic acid and ethyl linoleate *in vitro*, the wormicidal effects on *Clonorchis sinensis* were chronologically monitored in dose titration experiments. Excysted metacercariae were killed within a period of 31.0 ± 4.0 min, 149.3 ± 4.1 min and 207.0 ± 13.5 min with 100.0 mg, 0.1 mg and 0.001 mg linoleic acid, respectively. The time required for the linoleic acid to kill adult worms was 167.0 ± 0.8 min with 100.0 mg, 253.0 ± 0.8 min with 0.1 mg, and 277.0 ± 0.8 min at 0.001 mg titration. Clonorchicidal activity of ethyl linoleate was relatively delayed as death was observed within 263.3 ± 2.9 min, 286.0 ± 0.5 min, and 318.0 ± 0.8 min for 100.0 mg/ml, 0.1 mg/ml and 0.001 mg/ml concentrations, respectively. The wormicidal effects observed with these pure anti-clonorchal substances were found to be similar to the biological activity of native products derived from the mucus of the fresh water fish.

Key words: *Clonorchis sinensis*, clonorchicidal activity, fresh water fish, linoleic acid, ethyl linoleate

INTRODUCTION

Only certain species of fresh water fish are the second intermediate host of *Clonorchis sinensis*, yet all may inhabit in the same water source. It is well known that the penetration of fish skin by cercariae of *C. sinensis* varies with host species. The life span and infectivity of *C. sinensis* metacercariae in fish bodies also varies according to the species of host (Chun, 1964a, b; Suzuki and Komiya, 1966; Rhee, 1973).

Chun (1964c) originally reported that *C. sinensis* cercariae were killed by the surface mucus on the carps, *Carassius carassius* and *Cyprinus carpio*. More recently, in a series of studies designed to analyze the effect of clonorchicidal substances in the mucus of fresh water fish, soluble factors were purified from the

body surface mucus of *C. carpio*, *C. carpio nudus* and *Tilapia mossambica* by silica gel column and thin-layer chromatography. Subsequently, the substances were characterized by UV, IR and NMR-spectroscopy, and identified as linoleic acid and ethyl linoleate (Rhee *et al.*, 1983b, 1984 & 1987a, b).

In the present study, *in vitro* experiments were conducted to assess the clonorchicidal activity of pure linoleic acid and ethyl linoleate on developmental stages of *C. sinensis*.

MATERIALS AND METHODS

Metacercariae of *C. sinensis* were collected by peptic digestion method from a false dace, *Pseudorasbora parva*, caught in the Nagdong River at Kimhae, Korea. Excysted metacercariae were then isolated using artificial intestinal

juice. *C. sinensis* adults were obtained from bile ducts of rabbits previously infected with the metacercariae.

One g of either linoleic acid or ethyl linoleate (chemically pure, Sigma Chemical Co., St. Louis, MO, U.S.A.) was diluted in ethyl ether in a ten-fold titration system, carried out in 1 ml volumes containing 100.0 mg, 10.0 mg, 1.0 mg, 0.1 mg, 0.01 mg and 0.001 mg of each of respective substances. One ml of each concentration was transferred to a glass concave dish and the solvent allowed to evaporate. After the addition of 1 ml normal saline solution (0.85% NaCl) containing 10 fresh excysted metacercariae or adult worms, the viability of the respective developmental stages was assessed by direct examination with a stereomicroscope. Viability was determined on the basis of motility following the addition of 2 ml saline solution to each dish when the parasites were near death. Each experimental treatment was conducted in triplicate. Similarly prepared control groups (no linoleic acid or ethyl linoleate additives) were included.

RESULTS

Clonorchicidal effects of linoleic acid on excysted metacercariae: The clonorchicidal activity of linoleic acid was observed on the metacercariae *in vitro*. The mean life span following treatment was 31.0±4.0 min in 100.0 mg linoleic acid, 68.3±7.0 min in 10.0 mg, 120.0±4.4 min in 1.0 mg, 149.3±4.1 min in 0.1 mg, 176.6±5.2 min in 0.01 mg and 207.0±13.5 min in 0.001 mg, respectively.

As shown in Fig. 1, there was a direct inverse correlation between the concentration of linoleic acid and the life span of the metacercariae. The relationship was linear, and in agreement

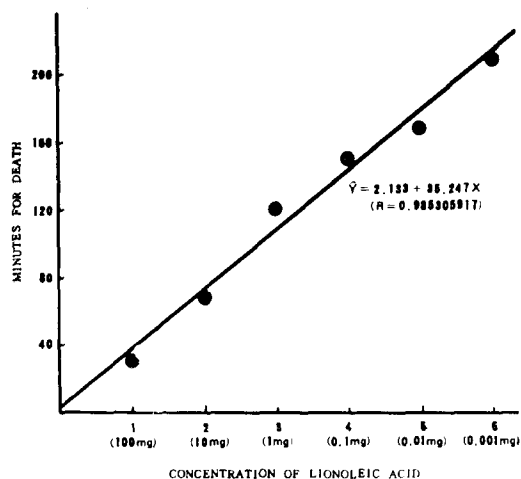


Fig. 1. Correlation of clonorchicidal activity with the concentration of linoleic acid.

with the linear equation $\hat{Y}=2.133+35.247X$, showing that the concentration (X) was a parameter ($R=0.9853$). The mean life span of the untreated metacercaria controls was 465.3 ± 28.5 min.

Clonorchicidal effects of linoleic acid and ethyl linoleate on adult worms: The clonorchicidal activity of linoleic acid and ethyl linoleate was observed on adult worms *in vitro*. Times for worm death in the linoleic acid-treated group are shown in Table 1. Mean life spans for the ethyl linoleate-treated group were 263.3 ± 2.86 min in 100.0 mg ethyl linoleate, 279.0 ± 0.81 min in 10.0 mg, 284.1 ± 0.81 min in 1.0 mg, 286.0 ± 0.47 min in 0.1 mg, 300.6 ± 1.24 min in 0.01 mg, and 318.0 ± 0.81 min in 0.001 mg, respectively.

The correlation between the concentration of the substances and the life span of adults in both groups was similar to that found with metacercariae. Again, the correlations were linear and conformed with linear equations $\hat{Y}=148.11\pm 22.0X$ ($R=0.9772$) in the linoleic

Table 1. Clonorchicidal effects of linoleic acid on the adult of *C. sinensis*

Amount of linoleic acid (mg)	100.0	10.0	1.0	0.1	0.01	0.001	Control
Minutes for death of worms	167.0 ^a ±0.816	189.6 ±1.24	214.0 ±0.816	253.0 ±0.816	250.0 ±0.816	277.0 ±0.816	633.3 ±11.95

^a mean±standard deviation

acid-treated group and $\bar{Y}=254.46 \pm 9.73X$ ($R=0.9611$) for the ethyl linoleate-treated parasites, demonstrating the concentrations (X) were parameters. Meanwhile, the mean life span of the control adult worms was 633.3 ± 11.95 min.

DISCUSSION

As part of observations on the suitability as second intermediate hosts, previous studies (Rhee *et al.*, 1983a & 1987b) involved natural and experimental infections of *C. carpio nudus* and *T. mossambica* with *C. sinensis* cercariae. Rhee and coworkers found that these fishes were not suitable intermediate hosts for *C. sinensis*. Moreover, Rhee *et al.* (1982) observed a large number of clavate cells in the epidermis of *C. carpio nudus*, and suggested that these cells limit the infectivity of *Clonorchis* metacercariae.

In preliminary experiments designed to clarify innate defense mechanisms of fresh water intermediate hosts to the *Clonorchis* parasite, clonorchicidal substances were purified from crude ethyl ether extracts from the mucus of *C. carassius*, *C. carpio*, *Parasilurus asotus*, *Ophicephalus argus* (Rhee *et al.*, 1979; 1980) and subsequently from *C. carpio nudus* and *T. mossambica* (Rhee *et al.*, 1983b & 1987b) by silica gel column and thin-layer chromatography. The substances were later identified as linoleic acid in *C. carpio nudus* and *T. mossambica*, and ethyl linoleate in *C. carpio* (Rhee *et al.*, 1984 & 1987a, b).

In the present study, the clonorchicidal effect of pure linoleic acid and ethyl linoleate *in vitro* confirmed the wormicidal activity of these substances. In general, the post-treatment life span of *C. sinensis* metacercariae and adults was inversely proportional to the concentration of linoleic acid/ethyl linoleate. These data are in agreement with previous reports which described the killing of metacercaria by chromatographically purified substances after 22 min in *C. carpio nudus* and 115 min in *C. carpio*, respectively (Rhee *et al.*, 1984 & 1987a),

Consequently, this study, together with previous works, strongly suggests the important role of linoleic acid and ethyl linoleate as chemo-protective factors in the innate resistance of certain fresh water fish to *C. sinensis* infection.

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＝국문초록＝

肝吸蟲에 대한 殺蟲性 物質에 관한 研究

VII. Linoleic acid 및 ethyl linoleate의 殺蟲 效果

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田이 1964年 잉어, 붕어 등의 體表粘液物質의 肝吸蟲에 대한 殺蟲作用說을 처음으로 발표한 以來, 몇가지 淡水魚類의 肝吸蟲에 대한 防禦機轉을 밝히기 위하여 著者등은 一聯의 實驗을 수행하였다. 그 결과 현재까지 잉어, 香魚, 鰱라피아의 體表 粘液의 ethyl ether 抽出物로부터 殺蟲性 物質을 純粹分雜하였고, 紫外線, 赤外線, 核磁氣共鳴分光分析을 통하여 잉어의 肝吸蟲 防禦物質은 ethyl linoleate, 香魚와 鰱라피아의 것은 linoleic acid 라는 것을 확인하였다. 이번에는 이들 物質이 肝吸蟲의 脫囊幼蟲과 成蟲의 生存에 미치는 영향을 *in vitro*에서 확인하기 위하여 다음과 같은 실험을 시도하였다.

이들 物質 1 g씩을 ethyl ether로 各各 10倍系列 稀釋하면 그 1 ml속에 100.0 mg, 10.0 mg, 1.0 mg, 0.1 mg, 0.01 mg, 0.001 mg의 이들 物質이 各各 함유하게 된다. 시계접시에 위의 희석액 1 ml씩을 취하여 溶媒를 완전히 증발시킨 다음 10마리의 肝吸蟲 脫囊幼蟲 또는 成蟲을 함유하는 生理食鹽液 1 ml씩을 넣고 직접 접촉시켜 脫囊幼蟲 또는 成蟲의 脫囊과정의 經時的으로 관찰하였다. 한편, 對照로서 시계접시에 ethyl ether 1 ml씩을 취하여 완전히 증발시킨 다음 生理食鹽液과 脫囊幼蟲 또는 成蟲을 더하여 그 脫囊과정도 관찰하였으며 實驗 및 對照群 各各 3反復으로 실험을 수행하였다. 또, 脫囊幼蟲 또는 成蟲의 脫囊과정은 그 運動의 完全停止 및 蟲體의 弛緩을 起點으로 하였다.

Linoleic acid에 직접 접촉시킨 脫囊幼蟲은 100.0 mg에서 31.0±4.0分, 10.0 mg에서 68.3±7.0分, 1.0 mg에서 120.0±4.4分, 0.1 mg에서 149.3±4.1分, 0.01 mg에서 176.6±5.2分, 0.001 mg에서 207.0±13.5分에 모두 脫囊하였다. 즉, 이 物質의 濃度가 낮을수록 脫囊幼蟲의 수명이 길어지는 傾向이며, 이러한 變化는 直線的으로 나타나며 濃度를 函數로 한 $\hat{Y}=2.133\pm 35.247X$ 의 一次方程式에 잘 符合되는 傾向이었다($R=0.9853$). 한편, 對照群에서 脫囊幼蟲의 수명은 465.3±28.5分이었다.

Linoleic acid 및 ethyl linoleate에 있어서 成蟲의 수명과 濃度와의 相關關係도 脫囊幼蟲의 경우와 비슷한 傾向이었다. 즉 모두가 直線的인 상관관계를 보이며 linoleic acid에서 $\hat{Y}=148.11\pm 22.0X$ ($R=0.9772$), ethyl linoleate에서 $\hat{Y}=254.46\pm 9.73X$ ($R=0.9611$)의 一次方程式에 잘 符合되는 傾向이었다. 한편, 對照群에서 成蟲의 수명은 633.3±11.95分이었다.