Production and Deformation of *Clonorchis sinensis* Eggs during In Vitro Maintenance

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**Abstract**

*Clonorchis sinensis* is a carcinogenic human liver fluke. The present study monitored eggs produced by long-term maintained adult worms of *C. sinensis* to confirm their egg productivity in vitro. The worms from infected rabbits were incubated in vitro in 1 × Locke’s solution and broth media (RPMI-1640, DMEM and IMDM). Numbers of expelled eggs were counted sequentially and their morphological changes were monitored by microscopy after 1, 30, 60, and 90 days of cultivation. On the 1–3 days of cultivation, the eggs counted maximum 4,756 ± 202 eggs/worm/day in IMDM medium. The number of eggs gradually decreased less than 1,000 at 7–14 days and below 100 at 21 days but continued to pass eggs after 56 days in all media. Length of the eggs were reduced about 1 μm at 30 days, and the length/width ratio was maintained around 1.8 at 30 days but decreased to 1.7 at 60 days and 1.5 at 90 days. Faust-Meleney index (FMI) decreased as the cultivation duration increased and lowest FMI (562.9 ± 974.7) observed in IMDM media at day 90 (*P* = 0.001). Microscopic findings of the eggs recognized the miracidium in most of eggs at 60 days but not in those at 90 days. Instead, the eggs contained dark granules or vacuoles in the deformed shell at 90 days. Scanning electron microscopy revealed partial loss of wrinkles on the deformed egg surface and prominent abopercular knob. Eggs viability decreased as the cultivation progressed and showed significant positive correlation with FMI and length/width ratio. In conclusion, the cultivated worms pass only the eggs which are preformed in their uterus before cultivation. One gravid *C. sinensis* contains about 37,000 eggs in its uterus and produces about 4,000 eggs every day. The deformed eggs with FMI less than 7,000 and length/width ratio lower than 1.7 are non-viable.

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**Introduction**

Helminthes usually produce a large number of eggs to overcome the transmission barriers in nature. Production of enough number of eggs is essential for most flukes because they survive over 3 domains of hosts. The 3 host domains include the first snail intermediate host, the second intermediate host, and the definite host. Biological transition between the hosts is a physical or biological barrier for a fluke. When an adult fluke produces many eggs, only a few of the eggs may survive by successful invasion into the snail host, the first intermediate host. The infected snails shed cercariae into water after asexual reproduction cycles in their body, and the cercariae invade the second intermediate or definitive host to continue its life. Egg production is a fundamental biological function for reproduction of flukes.

*Clonorchis sinensis* is a liver fluke of human causing clonorchiasis, which is prevalent in East Asia. Clonorchiasis is now classified as one of food-borne neglected tropical diseases. Since cholangiocarcinoma is a serious complication, clonorchiasis is a major health concern in endemic areas [1]. As egg production is an important process for a fluke’s biology and detection of eggs is a base of diagnosis, several studies observed egg productivity of *C. sinensis*. It has been reported that *C. sinensis* produce about 4,000 eggs/worm/day in humans, 2,400 eggs/worm/day in cats, and 1,600 eggs/worm/day in guinea pigs [2]. However, it is still unknown that *C. sinensis* may produce their eggs by in vitro cultivation.

Recently adult worms of *C. sinensis* were in vitro maintained long in broth media [3]. During the cultivation, the worms passed many eggs. The present study counted the eggs and observed morphological changes of eggs sequentially to investigate whether the worms are able to produce new eggs in broth media and whether they are viable.

**Materials and Methods**

**Ethics Statement**

The animal experiment was reviewed and approved by the institutional animal care and use committee of Seoul National University (2010).

**Collection of Adult Worms of *C. sinensis***

Metacercariae were collected from naturally infected fish *Pseudorasbora parva* according to the method described by Li et al. [4]. The collected metacercariae were preserved in cold (4°C) 1 × PBS with antibiotics until use. The metacercariae were introduced to male New Zealand white rabbits and adult *C. sinensis* worms were recovered as described in our previous study [3].
Table 1. Egg counts of *C. sinensis* daily produced by one adult worm in different media during the cultivation.

| Solution/Media | No. of eggs/worm/day (mean ± SD) at different day(s) | 1 | 3 | 7 | 14 | 21 | 28 | 35 | 42 | 56 | Appx. Total* |
|----------------|-----------------------------------------------------|---|---|---|----|----|----|----|----|----|-------------|
| 1 × Locke’s     | 220 ± 44                                            | 1023 ± 64 | 2827 ± 433 | 1317 ± 113 | 143 ± 33 | 296 ± 34 | 128 ± 3 | 88 ± 8 | 194 ± 37 | 38940 ± 3443 |
| RPMI-1640       | 2887 ± 219                                          | 4480 ± 257 | 1142 ± 152 | 317 ± 50 | 217 ± 57 | 165 | 99 ± 10 | 22 ± 4 | 21 ± 4 | 36571 ± 1026 |
| DMEM            | 4273 ± 152                                          | 1964 ± 374 | 2529 ± 228 | 437 ± 64 | 234 ± 41 | 43 ± 7 | 40 ± 8 | 22 ± 4 | 11 ± 2 | 39705 ± 311  |
| IMDM            | 4756 ± 202                                          | 2853 ± 240 | 1107 ± 189 | 456 ± 99 | 116 ± 17 | 45 ± 12 | 10 ± 3 | 56 ± 4 | 37 ± 10 | 33881 ± 2254 |

SD = standard deviation.

*Approximate total number of egg estimated by assuming same count of egg during intervals.

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In vitro Cultivation of Adult Worms of *C. sinensis*

The fresh worms were distributed in a 6-well culture plate by 10 worms per culture well with 3 mL of test solution or media as described in the previous study [3]. All of the experiment procedures were performed in triplicates. The media included inorganic 1 × Locke’s solution and 3 broth media: Roswell Park Memorial Institute-1640 medium (RPMI-1640), Dulbecco’s modified Eagle’s medium (DMEM), and Iscove’s modified Dulbecco’s medium (IMDM) with penicillin 100 μg/mL and streptomycin 100 U/mL concentrations. The 1 × Locke’s solution contained NaCl 0.9 g, KCl 0.42 g, NaHCO₃ 0.2 g, and CaCl₂ 0.24 g (wt/vol) in 1 liter of distilled water. The broth media were purchased from the WelGENE (Seoul, Korea). The culture solution or media were refreshed in every 3 days. The worms were cultivated in a humidified incubator at 37°C in the presence of 5% CO₂. Some of this experiment procedure was shared with the in vitro cultivation of *C. sinensis* adults [3].

Table 2. Measurements of eggs obtained from different media after cultivation (n = 15).

| Parameters                  | Media           | Day(s) of cultivation |
|-----------------------------|-----------------|-----------------------|
|                             | 1   | 30 | 60 | 90 |
| Length (μm)                 | 1 × Locke’s     | 29.23 ± 1.36         | 27.73 ± 1.20 | 27.49 ± 0.48 | ND |
|                             | RPMI-1640       | 28.32 ± 0.97         | 27.88 ± 0.69 | 28.11 ± 2.59 | 22.84 ± 2.04 |
|                             | DMEM           | 28.92 ± 0.93         | 27.98 ± 1.17 | 26.43 ± 2.45 | 24.91 ± 2.61 |
|                             | IMDM           | 29.58 ± 0.73         | 27.98 ± 0.91 | 27.79 ± 0.89 | 23.99 ± 1.60 |
| Width (μm)                  | 1 × Locke’s     | 16.22 ± 0.65         | 15.14 ± 0.24 | 15.77 ± 0.32 | ND |
|                             | RPMI-1640       | 15.39 ± 0.40         | 15.40 ± 0.37 | 16.03 ± 0.95 | 15.81 ± 1.07 |
|                             | DMEM           | 15.44 ± 0.49         | 15.30 ± 0.58 | 16.21 ± 1.06 | 16.06 ± 0.95 |
|                             | IMDM           | 15.28 ± 0.29         | 15.90 ± 0.32 | 15.77 ± 0.74 | 15.31 ± 1.03 |
| Length/Width ratio          | 1 × Locke’s     | 1.81 ± 0.11          | 1.83 ± 0.08 | 1.74 ± 0.05 | ND |
|                             | RPMI-1640       | 1.84 ± 0.06          | 1.81 ± 0.08 | 1.76 ± 0.19 | 1.44 ± 0.06 |
|                             | DMEM           | 1.87 ± 0.07          | 1.83 ± 0.09 | 1.64 ± 0.20 | 1.56 ± 0.18 |
|                             | IMDM           | 1.94 ± 0.06          | 1.76 ± 0.07 | 1.77 ± 0.09 | 1.57 ± 0.12 |
| Faust-Meleney Index (FMI)   | 1 × Locke’s     | 7699.3 ± 707.4       | 6361.2 ± 382.2 | 6839.4 ± 299.6 | ND |
|                             | RPMI-1640       | 6719.0 ± 511.4       | 7251.8 ± 1124.5 | 6608.6 ± 267.6 | 57940 ± 1393.9 |
|                             | DMEM           | 6904.3 ± 542.9       | 6957.8 ± 1006.1 | 6557.9 ± 594.9 | 6443 ± 596.13 |
|                             | IMDM           | 6908.0 ± 301.6       | 6926.4 ± 721.1 | 7073.1 ± 342.1 | 5662 ± 974.7 |

ND = No data, FMI: Faust-Meleney index.

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Egg Counts Expelled by Adult Worms

The wells of the 6-welled culture plates were cleaned properly before the collection of eggs. The eggs expelled by the adult worms for 24 hours in different media were collected from the wells by washing 3 times with respective medium. Numbers of eggs were counted after 1, 3, 7, 14, 21, 28, 35, 42 and 56 days, and the numbers of eggs/worm/day were estimated.

Measurement of Eggs under Light Microscopy

Eggs collected at 1, 30, 60, and 90 days of incubation were randomly measured for their length and width (n = 15) with a microscope (Olympus, Japan) at ×400 magnification, 0.29 resolving power with a resolution of 1,360 × 1,024 pixels (digital image) using Image-Pro® Express software version 4.0.1. For better understanding the dimensions of eggs and comparison, eggs obtained from different solution and media at different duration were evaluated by the length and width ratio (L/W) as well as Faust-Meleney Index (FMI = L × W²; where L, length and W, width) [5]. The eggs were also observed for internal contents such as miracidium, vacuolated globules, dark mass as well as...
prominence of abopercular knob using light microscope with ×100 or ×400 or ×1000 magnifications.

Scanning Electron Microscopy

For scanning electron microscopy (SEM) of eggs, long-term cultivated worms from 1× Locke’s solution and different broth media were fixed in 10% neutral buffered formalin and in 1% osmium tetroxide (OsO4) at 4°C. After dehydration through graded series of ethanol (50%–100%), the specimens were dried using a critical-point dryer. The dried adult worms were mounted on aluminum stubs and their body was dissected to open upper uterine branches which were full of eggs. The uterine eggs were coated with gold-plated metal supports. Ultrastructures of the egg shell surface and abopercular knob were observed by scanning electron microscope ABT DS-130C, Japan with ×2000 or ×4000 magnification. Normal C. sinensis eggs from freshly collected worms were also evaluated by SEM for the comparison of abopercular knob.

Determination of Egg Viability

Viability of C. sinensis eggs was determined by trypan blue (0.4%) staining. Eggs from different solution and media were mixed with equal volume of trypan blue and kept in room temperature for about 10 minutes. The eggs then examined under light microscope and the unstained one was counted as viable. Prior to the experiment eggs were stained with different kinds of dye namely eosin Y (0.1%), lugol’s iodine (1%), methylene blue (0.01%) and trypan blue (0.4%) to select the best (Supporting Information: Materials and Methods; Figure S1).

Statistical Analysis

All the data analyzed using Microsoft office excel 2007 program. Student’s t-test with two tails was performed and the Pearson’s correlation coefficient was determined for comparison and difference at \( P < 0.05 \) was regarded as significant.

| 1× Locke's | RPMI-1640 | DMEM | IMDM |
|------------|-----------|------|------|
| **1 Day**  | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| **30 Days**| ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| **60 Days**| ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| **90 Days**| ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |

**Figure 1. Light microscopic changes of C. sinensis eggs in different solutions and media during cultivation.** Morphological view of eggs from day 1 to day 90 arranged from top to bottom. As the worm survived up to 60 days in 1× Locke’s solution there was no data for day 90. All the eggs showed typical morphology at day 1, however, at day 90 eggs showed atypical shape, shorter length, prominent abopercular knob along with interior dark granules (×400). Scale bar: 10 μm.

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Results

Egg Counts in Different Media
Egg counts varied depending on the media and duration of cultivation (Table 1). The worms in IMDM produced 4,756 ± 202 eggs/worm/day on day 1, which was the highest among the solution and media. At day 3, the worms in RPMI-1640 produced significantly more eggs of 4,480 ± 257 than those in other media (P < 0.001). In the inorganic 1× Locke’s solution, a different behavior of egg expulsion was noticed, starting from low count of 220 ± 44 at day 1 to a peak of 2,827 ± 433 at day 7. The egg counts decreased to 1,317 ± 113 after 14 days, and less than 200 after 28 days. Few worms had eggs in their uterus afterwards in all media.

Measurements of Eggs under Light Microscope
Table 2 summarized measurements of the discharged eggs in culture media. Since the worms survived up to 60 days in 1× Locke’s solution, data were available till day 60. The egg length decreased significantly at day 30 and 60 in 1× Locke’s solution and at day 30, 60, and 90 in RPMI-1640 and IMDM media (P < 0.001). Average width of the egg showed variability among the solution and media. In 1× Locke’s solution it showed significant reduction from 16.22 ± 0.65 at day 1 to 15.14 ± 0.24 at day 30 (P < 0.001), however, it was increased slightly in the broth media but not significant. The ratio of length and width of eggs decreased significantly during the cultivation among the broth media (P < 0.001). The eggs in the Locke’s solution showed a significant decrease of the length/width ratio from 1.81 ± 0.11 at day 1 to 1.74 ± 0.05 at day 60 of incubation as shown in Table 2 (P < 0.001). FMI value decreased significantly between day 1 (7699.3 ± 707.4) and day 30 (6361.2 ± 382.2) in case of 1× Locke’s solution and between day 1 (6908.0 ± 301.6) and 90 (5662.9 ± 974.7) in case of IMDM media (P = 0.001). RPMI-1640 and DMEM also showed a lower FMI value at day 90 compared to day 1, however, it was not significant (P = 0.064 and P = 0.203 for RPMI-1640 and DMEM respectively).

Microscopic Changes of the Eggs
Light microscopy observed gradual morphological changes in the eggs from day 1 to 90 (Figure 1). All of the eggs were clean and distinctive of normal configuration on day 1. A few eggs included vacuoles on day 30 and both of number and size of the vacuoles increased on day 60. Most of the eggs discharged in the media or in the uterus of the worms were full of vacuoles or dark granules in their deformed egg shell observed at day 90 as presented in Figure 2. SEM observed surface topography of the eggs collected from 1× Locke’s solution at day 60 and from other broth media at day 90. Partial loss of surface wrinkles was evident among the eggs observed in 1× Locke’s solution and different media (Figure 3). Most of the eggs at day 90 demonstrated long prominent abopercular knob (Figure 4).

Viability of the Eggs
Almost all the eggs were viable among the solution and media collected on day 1, however, viability decreased gradually according to the duration of cultivation (Table 3). On day 30 and 90 eggs from RPMI-1640 showed highest viability (79.1% ± 1.11% and 34.3% ± 0.4% respectively) but on day 60, highest viability (67.8% ± 0.7%) was observed in 1× Locke’s solution. The egg viability showed a significant positive correlation with FMI values (1× Locke’s: r = 0.987, n = 6, P < 0.001; RPMI-1640: r = 0.671, n = 8, P = 0.034; DMEM: r = 0.697, n = 8, P = 0.027 and IMEM: r = 0.803, n = 8, P = 0.008). Correlation was also significant for length and width ratio except for 1× Locke’s solution (RPMI-1640: r = 0.736, n = 8, P = 0.018; DMEM: r = 0.805, n = 8, P = 0.007 and IMEM: r = 0.949, n = 8, P < 0.001).
Discussion

Adult C. sinensis worms which were incubated in broth media produced more than 4,000 eggs/worm/day at the beginning. Thereafter, the egg counts decreased to just above 1,000 at day 7, and to 300–400 at 14 day. The counts became lower than 100 at 28 day in the broth media. The counts were similar to each other among 3 broth media. Contrary to this, the counts in the Locke’s solution were 220±44 and 1023±64 at day 1 and 3 respectively. These counts were lower than those in broth media at the beginning, but the worms produced more eggs at day 21 and later.

Figure 3. Surface ultrastructure of C. sinensis eggs by scanning electron microscopy. Eggs after 60 days of incubation in 1× Locke’s solution (A) and 90 days of incubation in RPMI-1640 (B), DMEM (C) and IMDM (D) culture media (×2000). Arrow head showed smoothness on the surface or loss of wrinkles. Scale bar: 10 μm.
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Figure 4. Abopercular knob of normal and cultured *C. sinensis* eggs. (A) Light microscopy showed an inconspicuous abopercular knob in a normal egg (×1000). (B) Very prominent abopercular knob in cultured worm’s egg (×1000). (C–D) SEM images of normal and cultured worm’s eggs respectively (×2000). (E) Magnified posterior portion of normal egg (×4000). (F) Posterior portion of cultured worm’s egg with distinct abopercular knob (×4000). Scale bar: 10 μm for A–D and 5 μm for E–F.
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The mature eggs which form the miracidium may survive long but aborted ones do not. The degeneration of inner materials might have induced deformity and reduction of egg volume. A study found deformed eggs when mature Echinostoma caproni gravid adults stored in Locke’s solution up to 4 months at 4°C [8]. One possible explanation for above changes may be improper quinone tanning which is associated to the process of egg shell formation [9]. Long-term in vitro cultivation may have affected this phenol-oxidase mediated enzymatic process resulting deformed egg shell [10]. Thus prolonged in vitro cultivation of *C. sinensis* may deform the eggs as well as their viability.

Mature eggs of *C. sinensis* contain a very inconspicuous abopercular knob. *O. viverrini*, a closely related liver fluke, also shows very small to slightly developed abopercular knob of the eggs [7]. In the present study, abopercular knob of the egg shell became more prominent in most of the eggs after 60 days of incubation. The appearance of such a prominent abopercular knob was obvious in most degenerated eggs. Krejcí and Fried [11] demonstrated that abopercular knob can be shallow or deeply infolded in the egg shell in case of *Echinostoma caproni* and *E. trivolvis* respectively. Infolding of abopercular knob surrounding shell was not observed in the present study. The prominent abopercular knob may be an extension of existed normal inconspicuous knob in response to the changing physiology of dying worms. Taken together, it is difficult for the flukes to produce normal viable eggs by in vitro cultivation.

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The surface structure of eggs, observed by SEM, appeared to be a suitable morphological feature for understanding status of the egg shell. Among opisthorchiids, *C. sinensis* egg surface is extremely sculptured to make interlaced wrinkles of varying height which helps the ability to accumulate natural aquatic fibers and to increase the possibility of contact with molluscan hosts [12,13]. In the present study, diminished surface wrinkle was observed by SEM of the aborted eggs. This diminished wrinking with the prominent abopercular knob may be an outcome of degenerating process of the egg shell.

The present study had a few limitations. There were no direct data of egg viability to compare with morphological changes. In vitro hatching of eggs can give accurate information to the egg viability, but that of *C. sinensis* eggs is still unable. Also data of morphology and physiology of the incubated worms were limited.

In conclusion, the cultivated *C. sinensis* worms in broth media pass viable eggs for 60 days which are preformed in their uterus during their survival in rabbits. The preformed immature eggs are unable to develop miracidium by in vitro cultivation. One mature *C. sinensis* adult contains about 37,000 eggs in the uterus and produces about 4,000 eggs daily. The deformed eggs of the FMI value less than 7,000 and length/width ratio lower than 1.7 are non-viable.

### Supporting Information

File S1 Determination of *C. sinensis* egg viability using different kind of dyes.

(DOC)

**Figure S1** Staining of *C. sinensis* egg for the determination of viability with different types of dye. The upper and lower rows showed viable and non-viable eggs respectively. Trypan blue (0.4%) and cosin Y (0.1%) selectively stained the non-viable eggs where as lugol’s iodine (1%) and methylene blue (0.01%) stained both viable and non-viable eggs. Scale bar: 10 μm.

(TIF)
Author Contributions
Conceived and designed the experiments: S-TH MHU. Performed the experiments: MHU. Analyzed the data: MHU S-TH YMB M-HC. Wrote the paper: MHU S-TH.

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