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Short Paper

Lethal effect of high temperatures on the eggs of *Fasciola hepatica*

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Summary

Fasciolosis is a cosmopolitan parasitic disease with considerable economic and public health importance. *Fasciola hepatica* is the major cause of fasciolosis in man and domestic animals. Although remarkable research works have been done around the optimum temperature and time required for miracidial development, little is known about the exact susceptibility of *Fasciola hepatica* eggs to high temperatures. In the present study, *Fasciola hepatica* eggs were initially incubated at 40°C, 45°C, and 50°C for various times (1, 2, 3, 4 and 5 h), followed by incubation at 28°C for 16 days. Miracidial formation was subsequently investigated on the 16th day of incubation. Even though the rate of miracidial formation in the control group was 52%, in the eggs incubated at 40°C for 1, 3, and 5 h, the miracidial formations were 51.4%, 42.4% and 39.9%, respectively, and these values in the group incubated at 45°C were 46%, 42.5% and 33.7%, as well. However, in the case of incubation at 50°C for 1, 2, 3, 4 and 5 h, these values were recorded as 1.96%, 0.57%, 0.07%, 0.00% and 0.00%, respectively. The results indicated that the eggs were susceptible to high temperatures and incubation of the eggs at 50°C for 4 h was enough to significantly inactivate the eggs and prevent miracidial formation ($P<0.001$). However, using the livestock faeces as fertilizer in many rural areas may lead to the contamination of pasture. According to the findings of this study, in the areas with a high prevalence rate of *Fasciola hepatica*, manure storage for a sufficient time or heating the livestock manure before use as fertilizer is strongly recommended.

Key words: High temperatures, Lethal effect, Eggs, *Fasciola hepatica*, Control

Introduction

Fasciolosis caused by *Fasciola hepatica* is one of the major diseases of livestock, causing considerable economic losses due to mortality, liver condemnation, reduced production of meat, milk, and wool, and expenditures for anthelmintics (Kleiman *et al*., 2007). In addition, fasciolosis is now recognized as an emerging human disease, as WHO has estimated that 2.4 million people are infected with Fasciola and a further 180 million are at risk of infection (Anon., 1995).

Eradication of fasciolosis is rarely a practical option and control needs to be aimed at the reduction of the disease (Torgerson and Claxton, 1999). Fasciolosis control is almost exclusively carried out by strategic applications of a wide number of effective anthelmintics (Sanchez-Andrade *et al*., 2001). Only triclabendazole is efficacious both against pre-adults in hepatic parenchyma and adults in the bile ducts (Boray *et al*., 1983; Perez *et al*., 2005). However, the effectiveness of triclabendazole represents problems of resistance (Fairweather and Boray, 1999;

Robinson *et al*., 2002). Use of resistant animals, management regimens, avoidance of contaminated pastures and soil drainage for snail control are non chemical methods for the control of fasciolosis (Brundson, 1980; Suhardono, 1996; Cabaret *et al*., 2002).

Fasciola hepatica eggs are susceptible to heat; therefore, heating animal manure is an alternative method for the control of fasciolosis. To establish effective control programs, it is important to obtain an estimation of how long the parasite eggs remain viable or survive at high temperatures.

The rate of development of the egg increases with temperature within a range of 10°C to 30°C. Thus, at 30°C the miracidial formation is completed in 8 days. Above 30°C, development is increasingly inhibited and at 37°C does not occur at all. Mortality increases the longer the eggs remain at 37°C, with 100% mortality being reached after about 24 days (Rowcliffe and Ollerenshaw, 1960).

This study was therefore designed to define the lowest temperature to make *Fasciola hepatica* eggs inviable in the shortest time.

Materials and Methods

Preparation of *F. hepatica* eggs

Gall bladders of sheep naturally infected with *F. hepatica* were obtained from abattoirs located in Shiraz, southwestern Iran. The gall bladders were taken to the laboratory within 2 h. The bile was aseptically transferred into glass cylinders and left to set for 30 min. The eggs were settled down and gathered at the bottom of the cylinders. The supernatant was then removed and the yielded eggs were washed several times using normal saline; the eggs were finally transferred into a dark container containing normal saline and stored at 4°C for further use.

Incubation of eggs at high temperatures

The eggs were transferred into 33 special small plastic containers (Supa industries, Iran) containing 5 ml dechlorinated tap water. There was a small hole in the cap of the containers. The containers were then separately incubated at 40°C, 45°C and 50°C. The incubation times were 1, 3 and 5 h at 40°C and 45°C and 1, 2, 3, 4 and 5 h at 50°C, and the experiments were performed in triplicate.

Incubation of eggs at 28°C

After incubation of eggs at high temperatures, in order to investigate the effect of heat on the miracidial formation of *F. hepatica*, all of the above heated eggs were incubated at 28°C for 16 days. At the same time, three containers containing non-heated eggs were also incubated at 28°C as control groups. The eggs were checked microscopically for miracidial formation on the 16th day of incubation. The results of observations (micracidial formation) for the control and 33 test groups are summarized in Tables 1, 2 and 3.

Statistical analysis

Results are presented as percent of eggs

Table 1: Miracidial formation inside the heated (40°C) eggs of *F. hepatica* on the 16th day of incubation at 28°C

Experiments	Heating time (h)	1	3	5	Control
1	Examined eggs	412	353	463	377
	Eggs containing miracidium	243	148	170	210
2	Examined eggs	365	496	516	760
	Eggs containing miracidium	188	211	218	378
3	Examined eggs	665	256	358	572
	Eggs containing miracidium	310	110	140	300
Total	Examined eggs	1442	1105	1337	1709
	Eggs containing miracidium	741	469	534	888
	Eggs containing miracidium (%)	51.4%[a]	42.4%[ab]	39.9%[b]	52%[a]

Different letters in each row show significant difference (P<0.05)

Table 2: Miracidial formation inside the heated (45°C) egg of *F. hepatica* on the 16th day of incubation at 28°C

Experiments	Heating time (h)	1	3	5	Control
1	Examined eggs	374	394	487	377
	Eggs containing miracidium	180	172	177	210
2	Examined eggs	357	394	271	760
	Eggs containing miracidium	159	169	98	378
3	Examined eggs	521	339	337	572
	Eggs containing miracidium	237	138	108	300
Total	Examined eggs	1252	1127	1135	1709
	Eggs containing miracidium	576	479	383	888
	Eggs containing miracidium (%)	46%^{b}	42.5%^{b}	33.7%^{c}	52%^{a}

Different letters in each row show significant difference ($P \leq 0.01$)

Table 3: Miracidial formation inside the heated (50°C) eggs of *F. hepatica* on the 16th day of incubation at 28°C

Experiments	Heating time (h)	1	2	3	4	5	Control
1	Examined eggs	262	216	382	189	215	377
	Eggs containing miracidium	11	2	1	0	0	210
2	Examined eggs	377	288	493	222	308	760
	Eggs containing miracidium	6	1	0	0	0	378
3	Examined eggs	330	193	565	380	533	572
	Eggs containing miracidium	2	1	0	0	0	300
Total	Examined eggs	969	697	1440	791	1056	1709
	Eggs containing miracidium	19	4	1	0	0	888
	Eggs containing miracidium (%)	1.96%^{b}	0.57%^{b}	0.07%^{b}	0%^{b}	0%^{b}	52%^{a}

Different letters in each row show significant difference ($P<0.001$)

containing miracidium. After arcsin transformation, comparison of miracidial formation between different hours at each temperature was performed by analysis of variance and Tukey's post hoc test. All analyses were done by SPSS software (version 11.3) and p-value less than 0.05 was considered statistically significant.

Results

The results of the microscopic observations of incubated eggs at 28°C are presented in Tables 1, 2 and 3. Miracidial formation in heated eggs at 40°C for 1, 3 and 5 h was 51.4%, 42% and 39.9%, respectively (Table 1). As shown in Table 1, miracidial development in heated eggs at 40°C for 5 h was reduced significantly compared to the control and 3 h groups ($P = 0.03$). However, the difference between the control and 3 h was not significant ($P = 0.08$). Also, the difference between miracidial formation in the heated eggs at 40°C for 1 h and the eggs of the control group was not statistically significant ($P = 1$). Data shown in Table 2 indicate that heating eggs at 45°C may reduce the miracidial formation inside the eggs, especially when the time of heating increased to 5 h. Miracidial formation in heated eggs at 45°C for 1, 3 and 5 h was 46%, 42.5% and 33.7%, respectively. The difference between miracidial formation inside the heated eggs and those in the control group was statistically significant as well ($P<0.01$). As shown in Table 3, heating the eggs at 45°C strongly reduced the miracidial formation, as miracidial formation in heated eggs at 50°C for 1, 2, 3, 4 and 5 h decreased significantly to less than 2% compared with 52% in the control group ($P<0.001$).

Discussion

Although remarkable research works have been carried out on the optimum

temperature and time needed for miracidial development of *F. hepatica* (Thomas, 1883a, b; Rowcliffe and Ollerenshaw, 1960; Ollerenshaw, 1971a; Al-Habbib and Grainger, 1983; Getting and Byrom, 1991; Alcaino *et al*., 1993; Claxton *et al*., 1999; Kleiman *et al*., 2007), little is known about the susceptibility or resistance of these parasite eggs to high temperatures.

Temperature is one of the most important factors influencing the successful completion of the cycle of *F. hepatica* (Andrews, 1999; Kleiman *et al*., 2007). Eggs recovered from freshly passed faeces are undeveloped and temperatures of about 23°C to 26°C are most favorable for embryonation. At these temperatures, eggs become fully developed within 2 to 3 weeks (Thomas, 1883a, b). *Fasciola hepatica* eggs may remain alive for long periods under low temperatures, but they are susceptible to heat (Boray, 1969; Luzon-Pena *et al*., 1994; Andrews, 1999). Eggs refrigerated at 2°C to 10°C for 2.5 years could remain viable, although undeveloped, and after being kept at room temperature, miracidial development can be resumed and hatching take place over a period of 14 days (Krull, 1934). These miracidia are able to infect snails (Boray, 1969). In contrast, *F. hepatica* eggs are susceptible to heat and 37°C will kill the eggs after about 24 days (Rowcliffe and Ollerenshaw, 1960). When the eggs of *Fasciola gigantica* were incubated at 37°C for 5 and 10 days, the hatchability decreased to 4% and 1.4%, respectively. Temperature fluctuation from 4-32°C had an inhibitory effect on embryo development (32.3%) as compared to the controls (63.9%) (Hassan *et al*., 2008).

The current study was aimed at determining the exact susceptibility of *F. hepatica* eggs to heat considering the degree of temperature and time of heating. Our results indicated that *F. hepatica* eggs are susceptible to temperatures above 40°C, so increasing the temperature and heating time may lead to an increase in the mortality of the eggs and 50°C for 4 h is enough to inactivate the eggs completely and prevent the miracidial formation.

Fasciolosis is an emerging, reemerging parasitic disease in many countries (Mas-Coma *et al*., 2005), and because of its economic and public health importance, wider attention has been paid to its prevention and control. The type of control program that can be recommended depends on local husbandry and climatic conditions together with socio-economic factors relating to the livestock owner. These factors will vary widely between temperate and tropical climates, as well as between farmers in industrial compared to less developed countries. Control strategies in any given year can be modified, if necessary, based on the prediction given by these models. The potential environmental impact on consumer acceptance of any control measure must also be taken into account (Torgerson and Claxton, 1999).

Eggs of *F. hepatica* can remain viable in faeces from 3 weeks to several months, according to various conditions and the time of year (Rowcliffe and Ollerenshaw, 1960). Eggs in moist faeces can survive for at least 10 weeks in the summer and 6 months in the winter (Ollerenshaw, 1971a). In many parts of the world, especially in rural areas, farmers use the livestock faeces as fertilizer. Many farmers spread manures straight onto the land after removal from the housing, either because of inadequate storage capacity or for greater convenience (Smith *et al*., 2001b). Use of animal faeces as fertilizer causes pasture contamination and plays an important role in the distribution of infection (Suhardono, 1996; Biffa *et al*., 2006). According to the findings of this study, keeping the livestock manure at 50°C for 4 h before its use as fertilizer, prevents pasture contamination of *F. hepatica* eggs. The easiest and cheapest way for heating animal faeces is manure storage for a sufficient time before it's spreading in grazing areas. Solid manure storage for at least one month could eliminate most pathogens (Kudava *et al*., 1998; Himathongkman *et al*., 1999). The temperature in a solid manure heap increases over 55°C within the main body of the heap (Nicholson *et al*., 2005). Therefore, in areas with a high prevalence of *F. hepatica*, storage of animal manure for a sufficient time can be considered an effective control measure for fasciolosis.

Sufficient temperatures for killing the eggs is only produced in the depth of storage

of large bodies of manure, but in small bodies of manure and also in the surface portion of stored manure or in drier parts of manure heaps, the temperature produced by biological process is not sufficient to inactivate the eggs (Nicholson *et al*., 2005; Suhardono and Copeman, 2006), therefore, as a final conclusion, it could be recommended to heat these kinds of manure before using them as fertilizer in grazing areas.

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