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## Immune Response in *Fasciola gigantica* Experimentally Infected Rabbits Treated with Either Carnosine or Mirazid®

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**Abstract:** The aim of the present study was to determine and compare the antibody (Ab) response against *Fasciola gigantica* in experimentally infected rabbits treated with mirazid or carnosine by ELISA. Six groups of five rabbits each, were examined (negative control, negative treated with carnosine, negative treated with mirazid, infected positive control, infected treated with carnosine and infected treated with mirazid). Each infected rabbit was infected with 20 *Fasciola* metacercariae. Anti-*Fasciola* IgG was weekly evaluated by ELISA, in each animal serum, along the period of experiment (14 weeks). After scarifying the all animals, each liver slices were examined for the determination of flukes burden. Results showed highly significant difference between the numbers of *Fasciola* worms in liver of infected treated and control positive rabbits. A reduction of 54.5% in worm burden was detected in the carnosine treated group. While, the results of mirazid treated group presented complete eradication of flukes. IgG response in experimentally infected and treated groups, either with carnosine or mirazid, was significantly higher than that of control infected group. Carnosine infected group showed the highest Ab response of all groups. However carnosine action was not as potent as mirazid although it raised the general defence of the animal (Ab) plus moderate eradication effect on the worm. Thus, a combination of both carnosine and mirazid might only be recommended in patients presenting other causes of diminished immunity in addition to *Fasciola* infection.

**Key words:** *Fasciola*, immunization, carnosine, mirazid, ELISA, control

### INTRODUCTION

Tropical fasciolosis, caused by *Fasciola gigantica*, is the most economically important helminth infection of ruminants in Asia and Africa (Spithill *et al.*, 1997). In adult cattle, the course of the disease is often between chronic to subclinical, whereas it is reported to be acute often with a high rate of mortality in young calves (Soulsby, 1982). Parasitological diagnosis of fasciolosis is, however, possible only after 13-14 weeks post-infection by demonstrating fluke eggs in faeces. By this time, major damages to the host hepatic system may have already occurred. *Fasciola* species represents a recognized unsolved agricultural problem responsible for marked world-wide economic losses that mounted to US\$ 3 billion per year (Boray, 1985), as well as causing a not insignificant number of human infections. The estimated number of people infected with fasciolosis is 2.4 million in 61 countries, including Egypt (Motawea *et al.*, 2001). The population at risk is 27.7 million and the number infected is at least 830, 000 (Haridy *et al.*, 2002). The flukicide triclabendazole is the most effective drug to control *Fasciola* (Suhardono *et al.*, 1991). However, the cost of treatment and the emergence of resistance to drug in sheep infected with *F. hepatica* suggest a need to develop more sustainable strategies.

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Myrrh and loeo gum resin were obtained from the stem of *Commiphora molmol* (family: Burseraceae). A tree that grows in northeast Africa and the Arabian Peninsula. The council of Europe included myrrh in the list of plants that is acceptable for use in foods (Council of Europe, 1981). Purified extract of myrrh from *C. molmol* tree (Mirazid®), a new herbal schistosomicidal and fasciolocidal drug has been licensed in Egypt in March, 2002. It was documented by many studies to be efficient and safe (Massoud, 1999a, b; Massoud *et al.*, 1998; El-Gohary *et al.*, 1999; Badria *et al.*, 2001; Gaballah *et al.*, 2001; Massoud *et al.*, 2001a, b; Motawea *et al.*, 2001; Shier *et al.*, 2001; Hegab and Hassan, 2003). It was also proved to act as a potent larvicide (Massoud and Labib, 2003). Further, it had been proved to be effective in the treatment of dicrocoeliasis in man and animals (Massoud *et al.*, 2003).

Carnosine, a natural dipeptide found in animal tissues, was reported to act as a beneficial regulator of many biological processes, intracellular calcium regulation, antioxidant, buffer action (Quinn *et al.*, 1992), protecting against protein modifications (Hipkiss and Brownson, 2000). The dipeptide was also proved to have anti-inflammatory and anti-*Schistosoma mansoni* actions (Soliman *et al.*, 2001) and modulating action on the immune response (Nagai and Suda, 1988; Silaeva *et al.*, 1992; Suzuki *et al.*, 2001). Soliman *et al.* (2002) showed the possible antigenic efficacy of carnosine against the individual antigens of the three different stages of *S. mansoni* in immunized rabbits.

Although host immune responses induced with helminthes parasites have been extensively studied yet, there is limited information on those to *F. hepatica*. Serological diagnosis is preferred since anti-fasciola Ab can be detected as early as 2 weeks post infection. This can facilitate early chemotherapeutic intervention (Hillyer, 1998). Immuno-diagnosis of parasitic diseases is mainly based on antibody detection (Fagbemi and Obarisiagbon, 1990). It could reveal both recent and current infections with early diagnosis. To obtain a reliable diagnostic method, many authors prepared crude antigens from whole worm (Hillyer *et al.*, 1987; Abdel-Rahman and Abdel-Megeed, 2000). Excretory/secretory products (Espino and Finaly, 1994; Ralston and Heath, 1995) have been also prepared. The serodiagnostic methods for the detection of antibodies are quite sensitive to detect the infection in the early stages and have been exploited for the diagnosis of fasciolosis (Zimmermann *et al.*, 1985; Sanitago *et al.*, 1986). Adult somatic extracts and Excretory-Secretory (ES) products of *F. gigantica* have been used in ELISA (Maleewong *et al.*, 1996, 1999; Youssef and Mansour, 1991) for serodiagnosis of human fasciolosis.

In the present study, the immune responses of the *Fasciola* infected rabbits treated with either Mirazid or carnosine were evaluated by using ELISA.

## MATERIALS AND METHODS

### Animals

Thirty male New Zealand white rabbits, each weighing approximately 2 kg, were obtained from Animal House, Research Institute of Ophthalmology, Cairo, Egypt. They were routinely examined for intestinal helminthes and ectoparasites.

### Parasites

*F. gigantica* metacercariae were obtained from Theodore Bilharz Schistosomal Biological Supply Program (SBSP) located in Cairo for experimental infection of rabbits. Adult worms, identified as *F. gigantica* based on criteria described by Sahba *et al.* (1972), were obtained from infected bovine livers collected from local abattoirs for preparation of antigen.

### Experimental Design

Thirty rabbits were divided into six groups of 5 animals per group.

- G<sub>1</sub> = Non-infected non-treated control
- G<sub>2</sub> = Non-infected treated with carnosine
- G<sub>3</sub> = Non-infected treated with mirazid
- G<sub>4</sub> = *Fasciola* infected non-treated
- G<sub>5</sub> = *Fasciola* infected and treated with carnosine
- G<sub>6</sub> = *Fasciola* infected and treated with mirazid

- Carnosine is a natural dipeptide found in animal tissues was orally injected in a dose of 100 mg day<sup>-1</sup> for 10 days for each rabbit.
- Mirazid ®, a new herbal fasciolicidal drug has been licensed in Egypt was orally given in a dose of 20 mg day<sup>-1</sup> for six days for each rabbit.
- Infected rabbits received oral 20 viable *F. gigantica* metacercariae at the same time of treatment.

All rabbits were necropsied at 14 weeks after infection (end of experiment) for the determination of fluke burden. A general flow chart of all groups of animals and their treatment regime is seen in Fig. 1.

### Samples

#### Collection of Blood, Serum and Faeces

Individual blood samples were collected from all six groups at weekly intervals, up to 14 weeks. After separation of sera 0.01% merthiolate was added and stored at -20°C until use.

#### Faecal Samples

Coprological examination was carried out micro-scopically from 7th week post-infection, until end of the experiment by sedimentation method (Soulsby, 1965).

#### Preparations of *Fasciola* Somatic Antigen

Whole worm extract was prepared in Phosphate Buffer Saline (PBS) pH 7.2 as described by Oldham (1983). After 4-5 times washing of the live adult flukes in physiological saline, the flukes were homogenized (2 flukes mL<sup>-1</sup>) using a tissue homogenizer. The homogenate was then subjected to ultrasonication at maximum amplitude (peak to peak) for 10 min, two times, with an interval of 1 min using an Ultrasonicator (Misonix, USA). The preparation was centrifuged at 12,000 rpm for 45 min at 4°C. The supernatant was collected as somatic antigen of *F. gigantica* and its protein concentration was measured as described by Lowry *et al.* (1951).

#### Immunoassay (ELISA)

All the pervious mentioned anti-sera were conducted using standard ELISA protocol (Zimmermann *et al.*, 1985). Each well was coated with 10 µg mL<sup>-1</sup> of antigen. The sera were applied

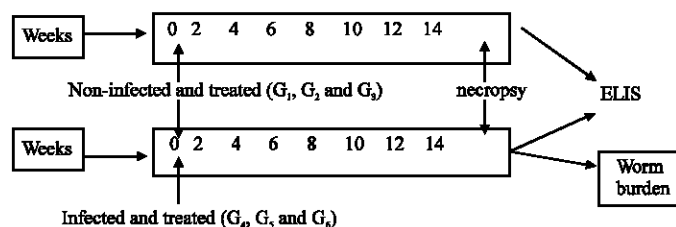


Fig. 1: Flow chart outlining by weeks the treatment and infection schedules

at 1:100 dilution in phosphate buffer saline. Anti-rabbit peroxidase 1:1000 (Sigma) and ABTS were applied as conjugate and substrate, respectively. The absorbance values were recorded at 405 nm. The cut off value calculated as double fold of O.D mean negative sera.

### Evaluations

After 14 weeks, the rabbits were sacrificed and the livers were dissected for the recovery of flukes (Wood *et al.*, 1995). The livers were then cut into slices approximately 1 cm thick and squeezed while in the saline solution; later the liver tissue was removed and the liberated parasites were collected after 30 min of sedimentation.

### Statistical Analysis

Experimental data were analyzed using ANOVA (Snedecor and Cochran, 1969). Significant difference (LSD) post- hoc (SPSS computer program).

## RESULTS

### Development of the Infection

*F. gigantica* eggs were never seen in the faeces of infected rabbits between 9 and 14 weeks post infection (pi). The mean number of flukes recovered from infected rabbits at necropsy is shown in Table 1. Recovered flukes in the infected groups were numbered. The statistically significant results were obtained in the groups infected-treated by carnosine or mirazid. There was significant differences in the mean fluke number between groups, but the percentage of flukes was higher in the G<sub>4</sub> (+ve control) than in the other group G<sub>5</sub> (infected and treated with carnosine) which revealing 54.5% worm burden reduction. However, no flukes were detected in G<sub>6</sub> (infected and treated with mirazid).

### Antibody Response to *F. gigantica*

The levels of IgG antibody were detected in sera from all groups of rabbits by ELISA. The data were shown in Table 2 and 3 and Fig. 2. The values of the G<sub>1</sub> (non-infected group non-treated) remained lower than the other two groups G<sub>2</sub> and G<sub>3</sub> which are clearly negatives throughout the experiment (Table 2, Fig. 2). The dynamics of IgG response was similar in all infected animals. In infected and treated groups G<sub>5</sub> and G<sub>6</sub>, specific IgG levels ascended quickly and by 3 weeks (pi) showed values higher than the positive control G<sub>4</sub> (Table 3, Fig. 2). The antibody levels of experimentally infected rabbits (G<sub>4</sub>, G<sub>5</sub> and G<sub>6</sub>) appeared at 2 weeks post pi. There was a steady increase, reaching a peak at 8-10 weeks pi, after which antibody levels decreased and became more or less stable until 12-14 weeks pi, when levels in all groups were still clearly positive. Significantly differences could be found between groups, the highly significant values were found in G<sub>5</sub> from the second week till the end of experiment.

In general, the reactivity of rabbit sera showed strong reactivity, highly significant values, in ELISA with *Fasciola* antigen in treated groups with mirazid and carnosine in comparison with non-treated.

Table 1: Effect of treatment of mirazid and carnosine on mean number of *F. gigantica* worms detected in liver of infected rabbits

Groups	Worm burden
Infected control G <sub>4</sub>	2.200±0.374 <sup>a</sup>
Infected and carnosine G <sub>5</sub>	1.200±0.200 <sup>b</sup>
Infected and mirazid G <sub>6</sub>	0.00 <sup>c</sup>

a, b, c: Means within the same column with different letter(s) are significant (p<0.05). G<sub>4</sub>: *Fasciola* +ve control, G<sub>5</sub>: Infected and treated with mirazid and G<sub>6</sub>: Infected and treated with carnosine

Table 2: The mean Optical Density (OD) variation in ELISA of 1/100 diluted sera of treated rabbits with mirazid or carnosine in ELISA

Groups	Weeks								Overall mean
	0	2	4	6	8	10	12	14	
-ve control G <sub>1</sub>	0.171±0.016	0.171±0.016	0.159±0.003	0.177±0.004	0.172±0.009	0.173±0.007	0.170±0.009	0.158±0.007	0.170 <sup>B</sup>
Non-infected treated with carnosine G <sub>2</sub>	0.187±0.005	0.199±0.017	0.231±0.004	0.293±0.020	0.290±0.003	0.248±0.009	0.251±0.007	0.245±0.020	0.243 <sup>A</sup>
Non-infected treated with mirazid G <sub>3</sub>	0.173±0.016	0.220±0.006	0.255±0.0180	0.255±0.0206	0.280±0.003	0.282±0.006	0.252±0.015	0.268±0.009	0.251 <sup>A</sup>
Overall mean	0.186 <sup>C</sup>	0.197 <sup>A</sup>	0.215 <sup>AD</sup>	0.242 <sup>A</sup>	0.247 <sup>A</sup>	0.234 <sup>AB</sup>	0.225 <sup>B</sup>	0.224 <sup>B</sup>	

Overall mean with different letter (in rows or column) are significantly different (p<0.05)

Table 3: The mean Optical Density (OD) variation in ELISA of 1/100 diluted sera of infected and treated rabbits with mirazid or carnosine in ELISA

Groups	Weeks								Overall mean
	0	2	4	6	8	10	12	14	
Infected control G <sub>4</sub>	0.169±0.014	0.260±0.017	0.426±0.003	0.534±0.036	0.634±0.012	0.642±0.021	0.635±0.038	0.524±0.010	0.478 <sup>F</sup>
Infected and carnosine G <sub>5</sub>	0.167±0.011	0.469±0.006	0.552±0.021	0.645±0.008	0.736±0.015	0.747±0.010	0.826±0.021	0.840±0.014	0.623 <sup>A</sup>
Infected and mirazid G <sub>6</sub>	0.189±0.007	0.366±0.016	0.408±0.009	0.552±0.021	0.645±0.008	0.699±0.007	0.747±0.010	0.759±0.014	0.546 <sup>E</sup>
Overall mean	0.1750 <sup>F</sup>	0.3651 <sup>F</sup>	0.4621 <sup>A</sup>	0.5771 <sup>A</sup>	0.6717 <sup>A</sup>	0.6959 <sup>B</sup>	0.7361 <sup>A</sup>	0.7077 <sup>B</sup>	

Overall mean with different letter (in rows or column) are significantly different (p<0.05)

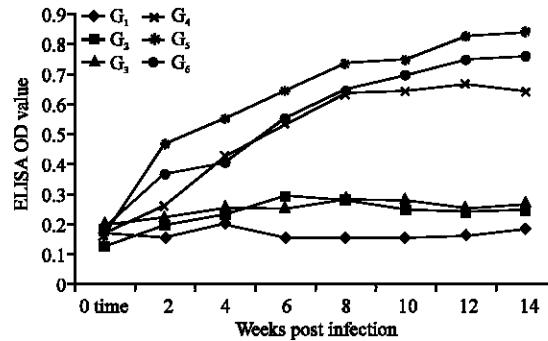


Fig. 2: Dynamics of the serum antibody response in *Fasciola gigantica* experimentally infected rabbits, either treated with carnosine or mirazid. G<sub>1</sub>: -ve control, G<sub>2</sub>: non-infected treated with carnosine, G<sub>3</sub>: non-infected treated with mirazid, G<sub>4</sub>: *Fasciola* +ve control, G<sub>5</sub>: infected and treated with carnosine and G<sub>6</sub>: infected and treated with mirazid

## DISCUSSION

In the present study, the statistically significant results were obtained in both infected-treated groups with either mirazid or carnosine. There was significant differences in the mean fluke number between groups, but the percentage of flukes was higher in the G<sub>4</sub> (infected non-treated) than in the other group G<sub>5</sub> (infected and treated with carnosine) which revealing 54.5% worm burden reduction. However, no flukes were detected in G<sub>6</sub> (infected and treated with mirazid). Mirazid® is a new drug proved to have high therapeutic efficacy as a trematocidal drug, particularly against schistosomiasis, fascioliasis and a cestodicide (El-Gohary *et al.*, 1999; Gaballah *et al.*, 2001; Massoud *et al.*, 2001 a, b; Motawea *et al.*, 2001; Shier *et al.*, 2001; Hegab and Hassan, 2003) Carnosine, a natural dipeptide found in animal tissues, was reported to act as a beneficial regulator of many biological processes; intracellular calcium regulation, antioxidant, buffer action (Quinn *et al.*, 1992), protecting against protein modifications (Hiplkiss and Brownson, 2000). The dipeptide was also proved to have anti-

inflammatory and anti *S. mansoni* actions (Soliman *et al.*, 2001) and modulating action on the immune response (Nagai and Suda, 1988; Silaeva *et al.*, 1992; Suzuki *et al.*, 2001). These different effects of carnosine might indicate the natural selectivity of dipeptide as a universal buffer correcting many derangements (Quinn *et al.*, 1992) for the benefit of the host. Immune suppression may be caused by parasite products as an immune evasion mechanism (Zambrano-Villa *et al.*, 2002). Recently, O'Neill *et al.* (2000) identified *F. hepatica* excretory/secretory products (*F. hepatica* cathepsin L proteinase), that was able to suppress interferon gamma production. *F. hepatica* has other immune evasion mechanisms such as rapid turnover of the glycocalyx and cleavage of the surface-bound immunoglobulins by secreted proteases that prevent the antibody-dependent cellular cytotoxicity. The parasite immune evasion mechanisms may allow chronic infection by preventing immune clearance of the parasite (Hawn and Jong, 2001; Dalton *et al.*, 2003).

The decreased parasite burden with treatment by carnosine explains the recovery of immune suppression in this study. This was documented by recent researches (Nutman, 2001; Pit *et al.*, 2001). In addition, carnosine modulating the immune response could find support in many previous researches. Carnosine had the ability to bind to macrophage and lymphocyte receptors stimulating their synthetic and secretory abilities, which may eventually lead to the activation of the natural systems of body immune resistance (Nagai and Suda, 1988; Silaeva *et al.*, 1992). It induced liberation of immune-modulator intermediates; cytokine and interleukin (Shimida *et al.*, 1999; Suzuki *et al.*, 2001). Carnosine modulation of immune response and decreasing apoptosis could, in part, be due to its antioxidant property (El-Moduem *et al.*, 2002). Carnosine scavenges Reactive Oxygen Species (ROS) and prevents accumulation of already formed peroxides (Salganik *et al.*, 2001; Boldyrev *et al.*, 2004). It also, facilitates the removal of deleterious proteins inactivated by carbonyl groups by forming protein-carbonyl-carnosine adduct (Hipkiss and Brownson, 2000; Hipkiss *et al.*, 2002). Further, carnosine intracellular defense mechanisms against Reactive Oxygen Species (ROS) was proposed to protect energy production and biochemical machineries of the cells (Soliman *et al.*, 2001). The authors proved normalization of adenylate nucleotides and adenylate energy charge. However carnosine action was not as potent as mirazid although it raised the general defense of the animal (Ab) plus moderate eradication effect on the worm. This matched the reported moderate healing ability of carnosine in other parasitic infection namely, *Schistosoma mansoni*. There was moderate anti-granuloma action, decrease in size of liver granuloma, diminution of its fibrotic and collagen content. However, enhancement of host immune response was shown by increased histostic and lymphocytic content (Soliman *et al.*, 2002).

*Fasciola*-ELISA test is widely applicable test sensitive for acute fasciolosis and its assessment of cure (Mousa, 2001). In the present study, the Ab levels of experimentally infected rabbits appeared at 2 weeks pi. There was a steady increase, reaching a peak at weeks 8-10 pi, after which Ab became more or less stable until 12-14 weeks pi. Significant differences could be found between groups, significant higher values were found in G<sub>5</sub> from the second week till the end of experiment. These data were in agreement with other previous researches (Kendall *et al.*, 1978; Zimmermann *et al.*, 1982; Mousa, 1994; Mbuh and Fagbemi, 1996), when antigens derived from adult worms were used. These findings were also in agreement with Massoud *et al.* (2004) who detected the mean value of Indirect Hemagglutination Assay (IHA) titer was 966 before treatment that decreased significantly after therapy in 88.6% of cases were still having high titers. Massoud *et al.* (2001b) reported marked decline of Ab three months after mirazid therapy using indirect fluorescent antibody test. In comparison with other fasciolicidal drugs, 90% of patients treated with praziquantel or bithionol had high antibody titer 3 months after treatment (Espino *et al.*, 1992) and 50% of those treated with triclabendazole and height titer 3 months after treatment (Hammouda *et al.*, 1995).

It is concluded that Mirazid® is, up till now, still the most safe and effective fasciolicidal drug at the clinical, parasitological and immunological levels. Although carnosine proved to be a save and

effective immunostimulant. These data inspires more hope for further research on prophylactic and therapeutic potencies of carnosine on *F. gigantica* infection, one of cosmopolitan human insults. Yet, alone it did not eradicate fasciola infection. Hence carnosine could be given in conditions associated with immune suppression. Another proposal is to further search treatment of different doses of carnosine combined with mirazid.

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