

## ***In vitro* effect of Albendazole and Albendazole sulphoxide on 70 and 60 kDa Heat Shock Proteins in *Echinococcus granulosus* protoscolices**

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**Abstract:** Heat shock proteins (HSP70 and HSP60) were immunohistochemically studied in *Echinococcus granulosus* protoscolices incubated *in vitro* with albendazole, or albendazole sulphoxide, or both, and under hyperthermic conditions. Antibody specificity against *E. granulosus* protoscolices was tested by immunoblotting technique. A significant loss of HSP70 and HSP60 immunoreactivity was observed after the benzimidazole carbamate treatment. *In vitro* hyperthermic treatments of *E. granulosus* protoscolices exhibited higher immunoreactivity levels than control protoscolices.

**Key words:** *Echinococcus granulosus*; HSP70; HSP60; albendazol; albendazol sulphoxide; Immunohistochemistry.

**Resumen:** Las proteínas del estrés o Heat Shock Proteins (HSPs) (HSP70 y HSP60) de protoescolex de *Echinococcus granulosus* fueron estudiadas mediante inmunohistoquímica tras incubación *in vitro* con albendazol, albendazol sulfóxido o la combinación de ambos, y bajo tratamiento térmico. La especificidad de los anticuerpos fue comprobada mediante inmunoblotting. Se observó un descenso en la inmunoreactividad de la HSP60 y HSP70 tras la incubación con los antihelmínticos. Por el contrario, los protoescolex incubados con tratamiento hipertérmico mostraron una mayor inmunoreactividad en ambas proteínas.

**Palabras clave:** *Echinococcus granulosus*; HSP70; HSP60; albendazol; albendazol sulfóxido; Inmunohistoquímica.

### **1. Introduction**

Echinococcosis is an helminthic zoonosis with a wide geographical distribution in the Mediterranean area as like as Minor Asia, East Africa, South of South America, Australia and so. This important parasitic disease is provoked by the (larval) metacestode stage of *Echinococcus granulosus sensu stricto* both in human and ungulated animals. Many attempts have been made to find an effective drug against this parasitic disease and, to date, benzimidazole carbamates are the most frequently used compounds. It is thought that the mode of action of the benzimidazole carbamates is to alter microtubule polymerization via a direct binding of these agents to parasite tubulin (Lacey, 1988; Criado

Fornelio *et al.*, 1990). An alternative hypothesis has been proposed by McCracken and Stillwell (1991), suggesting that benzimidazole carbamates may act as classic uncouplers in electron transport associated phosphorylation reactions in mitochondria.

The stress response is a general mechanism that protects cells and the entire organism from various deleterious effects. Heat-shock proteins (HSP) are mainly implicated in correct protein folding (Buchner, 1996). The role of HSPs in the complex network of host-parasite interactions is still not well understood (see review of Lathigra *et al.*, 1991). An increase in HSP production has been correlated with an increase in infectivity (Smekjal *et al.*, 1988), and parasite protection against host reaction has also been suggested (Lyons and Johnson, 1995; Salotra *et al.*, 1995).

HSP induction has been described after treatment with drugs, antibiotics, and other chemical compounds (see review of Macario, 1995). In view of

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the close interrelationship between infectivity and HSP expression shown by parasites, it was of interest to examine the effect that benzimidazole carbamates have on HSP expression. Considering the two possible mechanisms of action suggested for these carbamates (via tubulin synthesis and via mitochondrial phosphorylation reactions), we studied the expression of HSP70 in the cytoplasm, and HSP60 in mitochondria.

## 2. Materials and Methods

### 2.1) Source and maintenance of protoscolices

*Echinococcus granulosus* protoscolices were aseptically collected from liver hydatid cysts in sheep slaughtered at the municipal abattoir in Alcalá de Henares, Spain. Viability prior to testing was 95-99% as assessed by the methylene blue exclusion test and microscopic examination as previously described (Casado *et al.*, 1986). The organisms (1500 protoscolices/Leighton tube) were cultured in 10 ml of medium 199 (Flow Laboratories, Madrid, Spain), supplemented with 100 IU penicillin ml<sup>-1</sup> and 100 µg streptomycin ml<sup>-1</sup>. *In vitro* incubations were performed at 37 °C without changes of medium, as previously described (Casado *et al.*, 1989; Pérez-Serrano *et al.*, 1994).

### 2.2) Anthelmintic treatment

Albendazole (ABZ) and albendazole sulphoxide (ABZ.SO) (Smith Kline & Beecham Research Ltd, London, U.K.), either as separate compounds or mixed (1/1 v/v), were dissolved in 1:1000 dimethyl sulphoxide (DMSO) and added to the medium for a final concentration of 10 µg.ml<sup>-1</sup>. Protoscolices incubated with either culture medium alone or culture medium containing DMSO, served as controls. Each experiment was repeated 3 times. During incubation, the tubes were observed each day with an inverted light microscope, and samples of protoscolices were taken every 3 days for viability assessment using the methylene blue exclusion test.

When optical alterations in the protoscolices were detected (at 12 days, in the protoscolices incubated with ABZ and ABZ.SO), incubation was terminated and samples were prepared for immunohistochemical procedures.

### 2.3) Hyperthermic treatment

Control protoscolices (25000/Leighton tube) were incubated at 37 °C (control conditions) and 42 °C (hyperthermic treatment) in 10 ml of medium 199 (Sigma, St. Louis, Missouri) for 4 hr. The medium was then removed and protoscolices were treated as is described for immunohistochemical procedures.

### 2.4) Immunohistochemical methods

Protoscolices were fixed with 1.5% paraformaldehyde in 0.05 M Tris-buffer (pH 7.6) for 15 min. After fixation, protoscolices were processed for paraffin embedding. Sections of protoscolices were cut at 8 µm, deparaffinized, and then incubated with 3% H<sub>2</sub>O<sub>2</sub> for 20 min to inhibit endogenous peroxidase activity. Sections were subsequently incubated for 30 min in Tris buffer with 5% (w/v) non-fat powdered milk (blotto buffer) at room temperature in a humidified chamber to block non-specific binding. Without washing, sections were incubated overnight at 4 °C with the primary antibody diluted in Tris-buffer at pH 7.6. After 3, 5-min washes in Tris-buffer, the sections were incubated for 90 min at 20 °C with the peroxidase-conjugated secondary antibody diluted 1:200 in Tris-buffer and then rinsed 3 times with Tris-buffer. Peroxidase activity was determined with 0.03% 3,3'-diaminobenzidine (Sigma) in Tris-buffer with 0.01% H<sub>2</sub>O<sub>2</sub> for 10 min. Sections were then washed in distilled water, dehydrated in graded concentrations of ethanol, and mounted in DPX. Control sections were processed with a similar procedure, but the primary antibody was replaced with blotto buffer; these control sections showed no immunoreactivity. The monoclonal primary antibodies used in the present study were anti-HSP70 (Sigma, clone BRM-22) (1:600 diluted) and anti-HSP60 (Sigma, clone Lk2) (1:200 diluted in 0.1% Triton-X100 Tris-buffer).

HSP70 and HSP60 immunoreactivities were determined by densitometric analysis using a digital analysis system (MICROM, Madrid, Spain). The densitometric value for each protoscole section was divided by the area of the same section, which was also determined by digital analysis image system. Densitometric analysis was quantified in three different experiments. Twenty protoscolices were analyzed per sample (DMSO controls, ABZ, ABZ.SO, and both drugs in combined treatment), and for each method. Mean values were compared with the unpaired Student's *t*-test.

### 2.5) Immunoblotting

Control protoscolices were homogenized in 0.5 ml. of phosphate buffer saline (PBS) by sonication. This homogenized sample was centrifuged at 100000 g for 30 min at 4 °C. The supernatant was collected and the protein concentration was determined (Bradford, Bio-Rad). Samples (20 µg/well) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) stacking gels, containing 4 % and separating gels with 12 % acrylamide. Electrophoresis

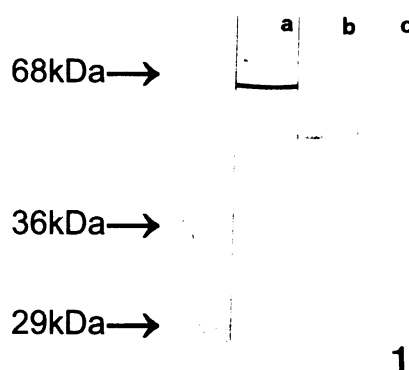


Fig. 1. HSP70 (a) and HSP60 (b) immunoblots. A control immunoblot without primary antibody is included in c. Molecular weights are indicated on the left.

was carried out at a constant current of 40 mA. Electroblood transfer from the polyacrylamide gels was performed as described by Towbin *et al.* (1979). The nitrocellulose blots were washed in PBS containing 0.05 % Tween-20, and incubated with blotting buffer for 1 hr. After the incubations, blots were tested with antisera. Primary monoclonal antibodies (Sigma) were anti-HSP70 (1/5000 dilution) and anti-HSP60 (1/1000 dilution), diluted in PBS. The peroxidase conjugated secondary antibody (Sigma) was used at 1/1.000 dilution. The peroxidase reaction was carried out of 0.05 M Tris buffer containing 0.03 % diaminobenzidine (DAB), and 0.001% hydrogen peroxide.

### 3. Results

#### 3.1) *Protoscolices viability*

Control protoscolices, after 12 days of incubation, remained 95% viable. ABZ or ABZ.SO incubations showed viability of 85% after the same period of incubation. In the combined treatment (with ABZ and ABZ.SO), the observed viability at the end of the incubations was 10%.

#### 3.2) *Antibody specificity*

Anti-HSP70 antibody showed better specificity than anti-HSP60 antibody. A single immunoreactive band was observed in westernblots for HSP70; however, 2 immunoreactive bands were observed in the anti-HSP60 immunoblots (Fig. 1). Immunoreactive bands were located in the expected position according to their molecular weights, and immunoreactive products were not observed in control blots incubated without primary antibody (Fig. 1). The same result was observed in control sections (Fig. 2).

#### 3.3) *HSP expression*

A significant reduction of HSP70 and HSP60 immunoreactivities was observed in treated protoscolices as compared to controls (Figs. 3a, b); no significant differences were observed among different treatments (ABZ, ABZ.SO, and ABZ+ABZ.SO). However, a significant increase of both HSP70 and HSP60 immunoreactivities was observed in heat-treated protoscolices (Figs. 4a, b). Some differences were observed when comparing immunostained sections for HSP70 and HSP60. HSP70 immunoreactivity was always stronger than that induced by HSP60. On the other hand, in control protoscolices, the heaviest immunostained structures were the sucker region and invagination channel; however, these were the structures that also exhibited the greatest loss of immunoreactivity after treatment. A strong HSP70 immunopositive ring around the calcareous corpuscles was very strong (Fig. 5). Sections of control and benzimidazole-carbamate treated protoscolices immunostained for HSP70 and HSP60 are shown in Figures 5 and 6.

### 4. Discussion

A significant loss of HSP70 and HSP60 immunoreactivities was observed in sections of *E. granulosus* protoscolices after the *in vitro* benzimidazole carbamate treatment. As far as we know, there are no data available concerning the effect of anthelmintics on HSP expression in cestodes, but it is well known that treatments with some chemical compounds, e.g., amino acid analogues, heavy metals, or anti-neoplastic chemicals, induce HSP expression in eukaryotic cells (Lindquist, 1986; Morimoto, 1991), especially if these compounds interfere with oxidative phosphorylation or electron transport (Lindquist, 1986). This is particularly interesting considering the hypothesis of McCracken and Stillwell (1991) that benzimidazole



Fig. 2. Control section of protoscolices without primary antibody (x575).

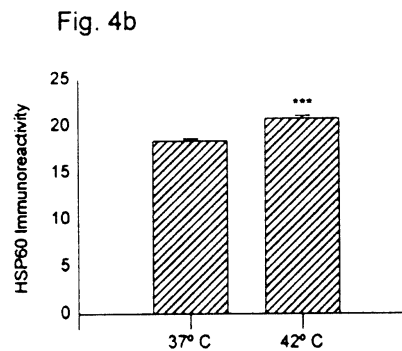
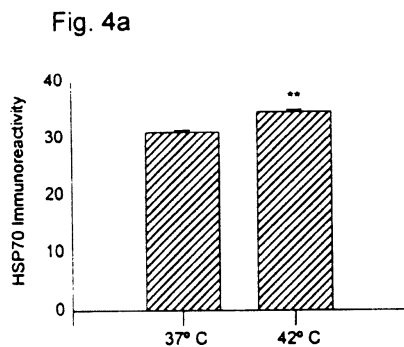
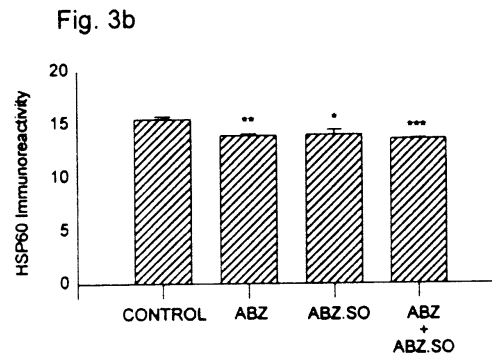
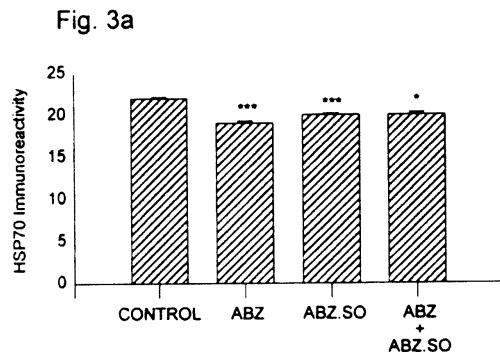


Fig 3 and 4: HSP70 (3a and 4a) and HSP60 (3b and 4b) immunoreactivities after benzimidazole carbamates treatment (3a and 3b) and hyperthermic treatment (4<sup>a</sup> and 4b). \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ , \*\*\*  $p < 0.001$

carbamates act like classic uncouplers in electron transport-associated phosphorylation reactions. In accordance with this hypothesis, an increase on HSP synthesis in *E. granulosus* protozoa could be expected after *in vitro* benzimidazole carbamate treatment. However, a significant reduction was seen in the present results, which may be correlated with the loss of the viability tested after the anthelmintic treatments even though differences were not much greater as compared with the viability of control protozoa. These results suggest that *in vitro* incubations with albendazole and albendazole-sulphoxide can provoke a general, deleterious effect on protozoa of *E. granulosus*, perhaps interfering with the expression of important proteins in a manner similar to HSPs or tubulin (Pérez Serrano *et al.*, 1995).

Stress-induced tolerance is a biological effect associated with HSPs expression; cells are protected from additional stronger conditions when they have previously experienced stress. This stress-induced tolerance may be caused by a condition different from that initially imposed on the cell. This type of tolerance is particularly interesting in the case of parasites

and hosts. The induction of HSPs in the host has been correlated with protection against infections (Nagasawa *et al.*, 1992; Himeno and Hisaeda, 1996). In this sense, HSPs could play an important role in developing effective defenses. In contrast, HSP expression in parasites has been correlated with virulence (Smejkal *et al.*, 1988). It may be that treatment



Fig. 5: Sections of protozoa HSP70 immunostained; (a) Control, (b) ABZ + ABZ-SO treated (x575).

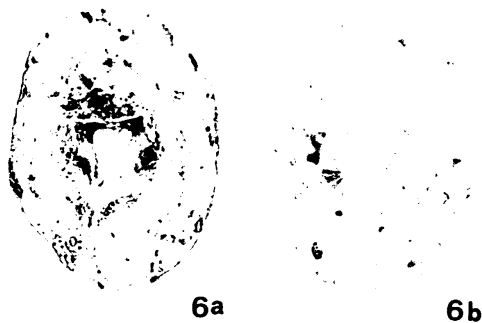


Fig. 6: Sections of protoscolices HSP60 immunostained; (a) Control, (b) ABZ treated (x575).

with benzimidazole carbamates could induce HSP expression and, subsequently, parasite tolerance which would then become more resistant. Fortunately, it seems that *in vitro* benzimidazole carbamate treatment does not induce HSP expression; on the contrary, a significant reduction of HSP expression is demonstrated in the present results. It is reasonable to speculate that culture conditions might modify the ability of HSP expression in *E. granulosus* protoscolices; however, heat stress demonstrated a clear induction of HSPs in cultured protoscolices.

### 5. Acknowledgments

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