

SHORT COMMUNICATION

Detection of HSP27-like molecules in the annelid *Enchytraeus japonensis* after exposure to extremely low frequency magnetic fields (50 Hz)**D Malagoli¹, M Marchetti², F Gobba²**¹*Department of Animal Biology, University of Modena and Reggio Emilia, Modena, Italy*²*Department of Public Health Sciences, University of Modena and Reggio Emilia, Modena, Italy**Accepted July 9, 2007***Abstract**

The immunocytochemical study performed on the annelid *Enchytraeus japonensis* revealed the presence of immunoreactive heat shock protein (HSP)27 molecules in different areas of the body. Positivity was observed in coelomocytes and in epithelial cells of the intestine wall. The exposition of the animals to 400 μ T magnetic fields (50 Hz) for 30 min provoked an increased immunoreactivity in some specimens, but after immunoblot experiments, no significant differences in the total content of HSP27-like molecules were found between exposed and non-exposed animals.

Key words: *Enchytraeus japonensis*; HSP27; immunocytochemistry; extremely low frequency magnetic field

Introduction

From the literature, it emerges that invertebrates are a suitable model to study the effects of extremely low frequency magnetic fields (ELF-MF). After the pioneering work performed by Goodman's group (1976) on the slime mold *Physarum polycephalum*, studies were subsequently carried out on the insects *Sciara coprophila* and *Drosophila melanogaster*, the molluscs *Cepea nemoralis* and *Mytilus galloprovincialis* and the nematode *Caenorhabditis elegans* (Kavaliers *et al.*, 1991; Goodman *et al.*, 1995; Junkersdorf *et al.*, 2000; Miyakawa *et al.*, 2001; Ottaviani *et al.*, 2002; Gobba *et al.*, 2003; Malagoli *et al.*, 2003, 2004, 2006).

MFs are able to influence a variety of biological systems (Goodman *et al.*, 1995; Del Re *et al.*, 2006; Malagoli *et al.*, 2006; Bernardini *et al.*, 2007), and several investigations have focused on the alterations provoked by MFs on the membrane ion channels and on the expression of heat shock proteins (HSPs). Regarding ion channels in invertebrates, we have demonstrated that the exposure of the mussel *M. galloprovincialis* to 50 Hz MFs ranging from 200 to 1000 μ T disturbs the reactivity of circulating cells (immunocytes) towards

N-formyl-Meth-Leu-Phe (fMLP) by altering potassium and calcium channel permeability (Ottaviani *et al.*, 2002; Gobba *et al.*, 2003). Also in the mollusc *C. nemoralis*, MFs provoke an alteration in calcium channel function (Kavaliers *et al.*, 1991). As other physiological stressors, MFs can provoke the expression of highly conserved genes coding for HSPs (Lindquist, 1986; Goodman and Blank, 1998). In the nematode *C. elegans*, exposure to MFs induces the expression of the *hsp70* (Goodman and Blank, 1998) and the *hsp16* genes (Miyakawa *et al.*, 2001), while an augmented expression of HSP70 and HSP90 was observed in the mussel *M. galloprovincialis* after repeated exposure to 400 and 600 μ T MF (Malagoli *et al.*, 2004).

In order to increase our knowledge of the possible effects of ELF-MF on the small HSPs in invertebrates, the present paper investigates the presence and expression of HSP27-like molecules in the annelid *Enchytraeus japonensis* following exposure to a 400 μ T intensity ELF-MF.

Materials and Methods**Animals**

Prior to experimental procedures, adult samples of *Enchytraeus japonensis* (Annelida, Oligochaeta, Enchytraeidae) were grown on agar medium for 1 month, as previously described in detail for *Enchytraeus crypticus* (Franchini and Marchetti, 2006).

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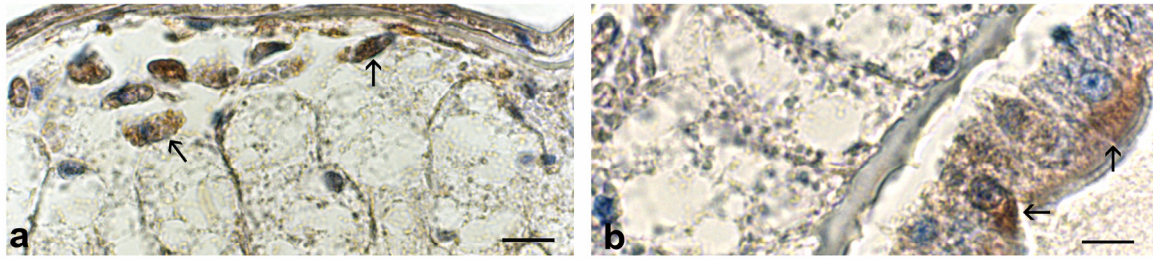


Fig. 1 HSP27-like molecules in coelomocytes (arrows) (a) and in epithelial cells (arrows) of the intestine wall (b) of *E. japonensis*. Nuclei are counterstained with hematoxylin. Bar = 10 μ m.

Exposure of animals to MFs

Each experiment was performed by placing 10 specimens for 2 h under a 50 Hz MF generated by two PVC coated coils (10x10 cm, 1400 windings, 0.2 mm \varnothing of copper wire) (IGEA, Carpi, MO, Italy) mounted horizontally 13 cm apart. The sinusoidal ELF MF intensity was controlled by an electronic power source (California Instruments, USA) connected to a computer and regulated by the "PGUI32" AC source control program (California Instruments, USA). ELF MF intensity and the temperature between the coils were constantly checked using a "7010 gauss/teslameter" (F.W. Bell, USA). MFs of 400 μ T were generated. Sham-exposed animals were maintained under the coils for the same time as the treated specimens, but in the absence of any current. All experiments were performed at room temperature. After 4 h of recovery, some animals were immediately fixed in Bouin's mixture and embedded in agar/paraffin, following Franchini and Marchetti (2006), while others were sacrificed for western blot analysis. Hematoxylin-eosin stain and immunocytochemical reactions were performed on 7 μ m longitudinal sections.

Immunocytochemical procedure

The immunocytochemical reaction was performed using avidin-biotin-peroxidase complex, as described in detail elsewhere (Franchini and Marchetti, 2006). Anti-HSP27 polyclonal antibody (pAb) (1:500) (Santa Cruz, CA, USA) was used as the primary antibody. Negative control experiments were performed by either omitting the primary antibody or substituting it with non-immune serum.

Western blot analysis for HSP27

Western blot analysis was performed on animals exposed to MFs and on sham-exposed animals (controls). Immediately after the exposure to MFs, the animals were frozen at -80°C for 30 min, then re-suspended in 95 μ l of sample buffer (12.5 % 0.5 M Tris-HCl pH 6.8, 10 % glycerol, 2 % SDS, 0.5 % 2-mercaptoethanol, 0.025 % bromophenol blue) and boiled for 4 min at 1200 rpm in a thermomixer (Eppendorf, Germany). Whole lysates were centrifuged at 13000xg at 4°C for 30 min, the supernatant was collected, and the protein

content quantified following Bradford's method (1976). Protein extracts were separated by 12 % SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and electrophoretically transferred onto PVDF membranes (0.2- μ m pore size). Western blots were performed using anti-HSP27 pAb (1:500) (Santa Cruz) and anti- β -tubulin monoclonal antibody (mAb) (1:1000) (Sigma, MO, USA) as primary antibodies. Immunoreactive bands were visualized using a NBT/BCIP detection system.

Densitometric analysis

Immunoblots were acquired using "Gel Doc XR" and digitally evaluated with the "Quantity One" software (BIO RAD Lab., Milano, Italy) and "ImageJ 1.32j" (Wayne Rasband, National Institute of Health, USA).

Statistical analysis

Statistical analysis of densitometric values was performed by ANOVA.

Results and Discussion

The pot worm *E. japonensis* has normally been used as a model to study the mechanisms of annelid regeneration (Myohara *et al.*, 1999, 2006). In the present research, we sought to verify if the animal could also be used as an indicator for ecotoxicological stress, particularly in areas subjected to MF irradiation. Previously, our and others laboratories have found interesting invertebrate models from different habitats that are very sensitive to MFs and HSP expression as a result of MF irradiation (Lindquist, 1986; Goodman and Blank, 1998; Miyakawa *et al.*, 2001; Malagoli *et al.*, 2004). The majority of these investigations examined HSP70 and 90, which are typically related to stress-response (Morimoto *et al.*, 1997), but did not evaluate the effects of ELF-MF on the small HSPs, including HSP27. However, relationships between ELF-MF and HSP27 could be of some interest in considering the role of these proteins in cytoskeletal dynamics (Dalle Donne *et al.*, 2001; Mounier and Arrigo, 2002), as well as with regards to the suggested involvement of some cytoskeletal components in determining ELF-MF effects (Gartzke and Lange, 2002). As far as the small HSPs in

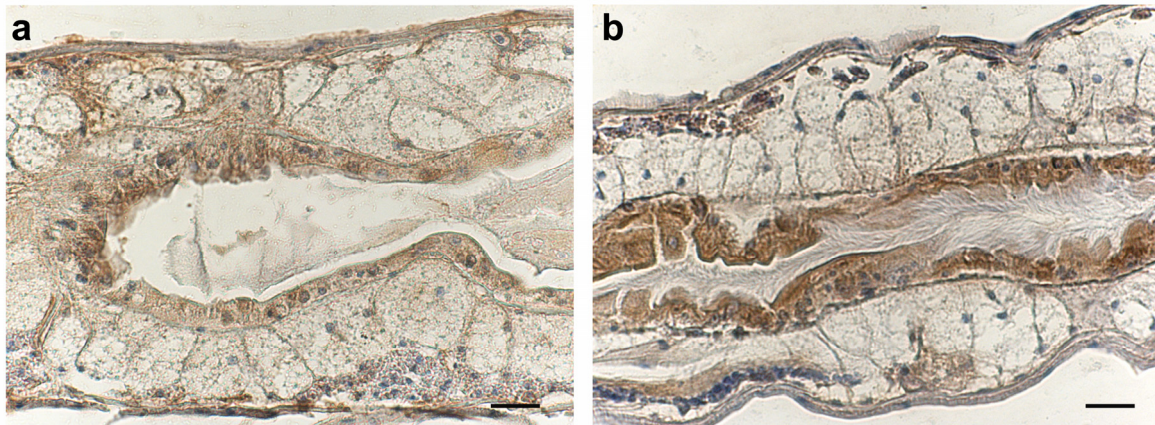


Fig. 2 Pot worm exposed for 2 h to 400 μ T ELF-MF shows an increased immunoreactivity to anti-HSP27 pAb (**b**) with respect to control (**a**). Nuclei are counterstained with hematoxylin. Bar = 20 μ m.

invertebrates are concerned, Miyakawa *et al.* (2001) demonstrated the presence of the *hsp16* gene in *C. elegans*. Working with *D. melanogaster*, Tanguay's group (2006) found that the family of small HSPs is composed of 4 components: HSP22, 23, 26 and 27, localized in different cell compartments, i.e. HSP22 in the mitochondria, HSP23 and HSP26 in the cytosol and HSP27 in the nucleus.

In *E. japonensis*, the HSP27-like molecules are distributed in the cytoplasm of coelomocytes and in epithelial cells of the intestine wall (Fig. 1). Even if in some specimens exposed to MFs, a higher immunoreactivity was detected compared to sham-exposed animals (Fig. 2), the hematoxylin-eosin staining did not reveal significant morphological modifications in the cells of treated animals. Furthermore, western blot experiments performed on protein extracts of the whole animal failed to evidence any difference between exposed and control specimens (Fig. 3).

This findings are in agreement with previous data from the bivalve mollusc *M. galloprovincialis*. The exposure of mussels to ELF-MFs in a range of 200-1000 μ T induced intensity-correlated effects on immunocyte functionality. However, at 400 μ T MFs only transient damage was observed, while the injury became permanent only after exposure to MF intensities ranging from 600 to 1000 T (Ottaviani *et al.*, 2002). Moreover, HSP70 and 90 were not induced in mussel immunocytes at 400 μ T after a single 30 min exposure (Malagoli *et al.*, 2004), a result that has also been confirmed by RT-PCR experiments (Malagoli *et al.*, 2006).

As small HSPs exert their cytoprotective role via antioxidant, antiapoptotic and actin-stabilizing properties during cell stress (Ciocca *et al.*, 1993; De Franco *et al.*, 2004; Franklin *et al.*, 2005), we can conclude that a single 2 h exposure to 400 μ T ELF-MF is not able to influence significantly cell integrity and HSP27 expression in the pot worm *E. japonensis*. This conclusion is based on western blot experiments performed on pooled animals. However, the immunocytochemical approach performed on single specimens revealed that some animals may be more sensitive to MF irradiation,

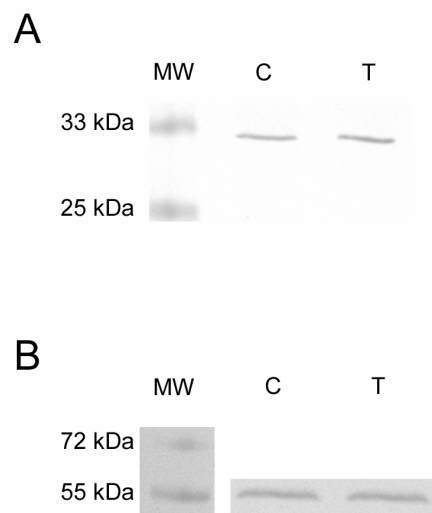


Fig. 3 Western Blot analysis of HSP-27 immunoreactivity in *E. japonensis* after 2 h exposure to 400 μ T ELF-MF (**A**). Immunoreactivity towards β -tubulin was adopted as loading control (**B**). MW = molecular weight standard; C = sham-exposed pot worms; T = exposed pot worms.

displaying a high level of HSP27-like material after the ELF-MF exposure.

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