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Expression Dynamics of HSP Genes in Hydatid Germinal Cells Exposed to Hydrogen Peroxide

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ABSTRACT: Cystic echinococcosis, caused by the larval stage of *Echinococcus granulosus*, is a chronic parasitic disease in which parasite tolerance to oxidative stress may facilitate long-term survival within the host. Heat shock proteins (HSPs) are conserved molecular chaperones that maintain proteostasis under adverse conditions. This study investigated the presence, sequence conservation, and oxidative-stress responsiveness of HSP60, HSP70-5 and HSP90-5 in *E. granulosus* G1, alongside the cytotoxic effect of H₂O₂ on G1 germinal cells. Target genes and germinal cells were obtained from hydatid cysts collected from naturally infected sheep livers. HSP genes were amplified by conventional PCR, sequenced, and compared with G1 reference sequences. Germinal cells were exposed to H₂O₂, and cytotoxicity and gene expression were assessed using MTT assay and RT-qPCR, respectively. All three HSP genes were detected in 100% of cyst samples and showed 100% nucleotide identity with corresponding G1 references. H₂O₂ induced concentration-dependent cytotoxicity in G1 germinal cells (IC₅₀ = 350 μM). H₂O₂ exposure significantly upregulated all investigated genes; HSP70-5 showed the strongest induction (187.56-fold at 200 μM; 238.92-fold at 350 μM), whereas HSP60 increased to 2.51- and 3.08-fold, and HSP90-5 to 3.21- and 3.92-fold, respectively. These findings indicate that HSP60, HSP70-5 and HSP90-5 are highly conserved in *E. granulosus* G1 and support their potential utility as diagnostic markers for this strain. Despite lacking major enzymatic antioxidant defenses, G1 germinal cells exhibited substantial oxidative-stress tolerance, underpinned by marked upregulation of HSP genes, particularly HSP70-5. Coordinated HSP-mediated responses may thus represent a key molecular basis for the prolonged persistence of *E. granulosus* G1 within the intermediate host.

INTRODUCTION

Cystic echinococcosis (CE) is an important zoonotic disease affecting both humans and animals and is caused by the larval metacestode stage of *Echinococcus granulosus*, a cestode belonging to the family Taeniidae that infects a broad range of domestic and wild mammals (1). Completion of the parasite's life cycle requires two mammalian host types, with domestic herbivores acting as intermediate hosts and canids serving as definitive hosts. Furthermore, humans can become accidental intermediate hosts following exposure to the infective stages of the parasite (2). Cystic echinococcosis has a worldwide distribution, occurring in regions of North and South America, Asia, Australia and Africa; furthermore, it remains endemic in several areas particularly in North African countries (3). The complex biology of *E. granulosus* and the chronic nature of CE present substantial challenges for effective disease management. These challenges include difficulties in early diagnosis, limited therapeutic options, differences

in cyst size and anatomical localization; the ability of the parasite to tolerate oxidative stress, the absence of an effective vaccine, immune evasion and the emergence of drug resistance (4). In addition, the remarkable adaptability of *E. granulosus* enables it to survive under unfavorable environmental and physiological conditions, thereby facilitating its persistence within the host (5). Furthermore, successful survival requires the parasite to overcome multiple host-derived stresses and immune defenses. Among the mechanisms contributing to this adaptation are heat shock proteins (HSPs), which play important roles in protecting parasite cells against adverse conditions and cellular stress (6). Consequently, understanding the molecular mechanisms underlying HSP-mediated stress tolerance may provide important insights into parasite survival and persistence, and may additionally offer practical value beyond basic physiology.

HSPs are evolutionarily conserved molecular chaperones that are essential for maintaining cellular protein homeostasis under both physiological and stressful conditions. They facilitate the proper folding of newly synthesized proteins, assist in the refolding of damaged proteins, prevent inappropriate protein aggregation and contribute to the stabilization and restoration of cellular proteins under adverse conditions (7). Furthermore, major HSP families, including HSP60, HSP70 and HSP90, have been identified in *E. granulosus* and are associated with the parasite's response to physiological and environmental stress, suggesting an important role in its adaptation and survival (8). Their importance may become particularly evident under oxidative stress, during which cellular proteins and other macromolecules are susceptible to oxidative damage. Hydrogen peroxide (H_2O_2) is a widely used experimental inducer of oxidative stress because of its ability to generate reactive oxygen species (ROS) and promote oxidative damage in biological systems (9). In mammalian cells, this cytotoxic effect is well documented; for instance, normal human pulmonary fibroblasts show a half-maximal inhibitory concentration (IC_{50}) of approximately 50 μM following 24-hour exposure to H_2O_2 (10). In *E. granulosus*, oxidative challenge induced by H_2O_2 can similarly disturb cellular homeostasis and damage essential macromolecules, thereby imposing significant stress on parasite cells (11). Notably, however, the parasite's antioxidant defense differs markedly from that of many other organisms, including its mammalian hosts. Conventional catalase and glutathione peroxidase activities have not been detected in *E. granulosus*, leaving the thioredoxin peroxidase system (EgTPx) as the principal enzymatic route for peroxide detoxification; experimental silencing of EgTPx has been shown to significantly reduce parasite viability under H_2O_2 -induced oxidative stress, confirming its central protective role (12). Moreover, thioredoxin peroxidase secreted by *E. granulosus* has been implicated not only in oxidative defense but also in modulation of host macrophage responses, suggesting that this molecule may contribute to both parasite survival and host-parasite interaction (13).

Nevertheless, antioxidant detoxification alone may not be sufficient to prevent the downstream consequences of oxidative stress, particularly oxidative damage to cellular proteins. Protein oxidation can result in misfolding and aggregation, and consequently, molecular chaperones such as HSPs may provide an additional layer of cellular protection by maintaining protein homeostasis despite the parasite's comparatively limited enzymatic antioxidant repertoire. Consistent with this concept, proteomic analysis of *E. granulosus* demonstrated that H_2O_2 exposure increased the abundance of HSP70 and altered several proteins associated with oxidative-stress responses and protein quality control (14). Beyond their protective function, HSP70 antigens of *E. granulosus* have also shown considerable immunodiagnostic relevance, being recognized by IgG, IgG4, and IgE antibodies in the sera of patients with cystic echinococcosis (15), and have already been incorporated into a rapid colloidal-gold immunochromatographic strip for field detection of the parasite in sheep (16). In related helminth systems, HSP70 orthologues have further been shown to elicit protective immunity through dendritic-cell activation, supporting their candidacy as vaccine antigens (17). Therefore, investigating HSPs in the context of oxidative stress may provide important insight not only into the mechanisms that enable *E. granulosus* to tolerate adverse conditions and persist within the host, but also into their potential value as diagnostic and vaccine targets.

Accordingly, the present study investigated the presence and sequence conservation of HSP60, HSP70-5 and HSP90-5 in hydatid cysts and subsequently evaluated their transcriptional responses to H_2O_2 -induced oxidative stress in G1 germinal cells. By integrating gene detection, sequence analysis, and expression profiling under oxidative stress, this study aimed to clarify the potential contribution of these conserved HSP genes to the stress-response mechanisms and long-term survival of *E. granulosus* G1, while also assessing their value as candidate molecular markers for strain identification and as potential targets for future diagnostic and vaccine-oriented research.

MATERIAL AND METHOD

Sample collection

Between November 2022 and May 2023, hydatid cyst specimens were retrieved from 60 infected sheep livers. The samples were collected from central abattoirs located in Basrah and Thi-Qar. Hydatid fluid and protoscolices were subsequently aspirated from the isolated cysts in accordance with previously reported protocol (18,19)

DNA extraction and conventional PCR

Genomic DNA was extracted from protoscolices and germinal layers obtained from hydatid cysts collected from the livers of naturally infected sheep using the G-sync™ DNA Extraction Kit (Geneaid, Taiwan). DNA concentration was determined using a Nano Drop spectrophotometer. Conventional polymerase chain reaction (PCR) was subsequently performed to amplify the HSP60, HSP70-5 and HSP90-5 genes. The HSP genes were identified based on the whole genome sequence (NW_020170395.1) and then specific primers were designed in the current study using Primer3. The PCR reactions were performed in a final volume of 25 μ L, consisting of 5 μ L of genomic DNA, 1 μ L of each primer, 12.5 μ L of Master Mix, and 5.5 μ L of nuclease-free water. The HSP60 gene was amplified using HSP60_F (5'-TCTCGTCATTGTTGCTGAGG-3') and HSP60_R (5'-GTGTAAGCGGGAGTTTCGAG-3'), producing a 727-bp amplicon. The HSP70-5 gene was amplified using HSP70_5F (5'-CAAACCTGTGCCTCCTTCTC-3') and HSP70_5R (5'-GTTTGCAGGCTGGCTTTTAG-3'), producing a 577-bp amplicon, whereas the HSP90-5 gene was amplified using HSP90_5F (5'-AGCTCTGCACCCACTGATCT-3') and HSP90_5R (5'-TCAGGAGTTTGTGCTGTTGC-3'), producing a 902-bp amplicon. PCR amplification was performed under the following conditions: an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 10 min. The amplified products were separated by electrophoresis on 1.5% agarose gel at 75 V for 45 min and visualized under UV illumination at 254 nm using a 1000-bp DNA ladder. Selected PCR products were submitted to Macrogen (Republic of Korea) for nucleotide sequencing. The obtained nucleotide sequences were subsequently analyzed and aligned using MEGA X with Maximum Likelihood method.

Germinal Cell Culture and Maintenance

Germinal cells (4×10^6) of *E. granulosus* sheep strain G1 were cultured and routinely maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies) and 100 U/ml penicillin–streptomycin. Cultures were maintained in 25-cm² tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO₂. When cultures reached approximately 80–90% confluence, cells were subcultured. The spent medium was removed, and the monolayer was washed twice with 2 mL phosphate-buffered saline (PBS). Cells were subsequently detached using 1 mL trypsin–versene solution and incubated at 37°C for 2–5 min. The detached cells were resuspended in complete culture medium and used for subsequent experiments. Cell concentration and viability were determined by the trypan blue exclusion method using a haemocytometer, as previously described by (20). Cells were subsequently maintained under the aforementioned culture conditions or preserved at 20– °C when required for downstream analyses.

Effect of H₂O₂ on cell proliferation

The cytotoxic effect of H₂O₂ on the proliferation of G1 strain germinal cells was evaluated using the MTT assay. Cells (1×10^4 cells/well) were seeded in 96-well plates containing 100 μ L of RPMI 1640 medium and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. After formation of a monolayer, the culture medium was replaced with serum-free RPMI 1640 containing H₂O₂ at 0, 200, and 350 μ M. Cells were subsequently incubated under the same conditions for 72 h. Each concentration was tested in triplicate, and the experiment was independently repeated three times. Following treatment, cell viability was determined using the MTT assay. Briefly, the culture medium was replaced with 90 μ L of serum-free medium containing 10 μ L of MTT solution (5 mg/mL in PBS), followed by incubation at 37°C for 2–4 h. The resulting formazan crystals were dissolved by adding 100 μ L of dimethyl sulfoxide (DMSO) followed by incubation at 37°C for 20 min. Absorbance was measured at 490 nm using a microplate reader. The half inhibitory concentration (IC₅₀) of H₂O₂ was determined from the concentration response relationship obtained from three to five biological replicates. Inhibition percentages were calculated for each H₂O₂ concentration and the IC₅₀ value was estimated using GraphPad Prism version 7. Cell viability and inhibition rates

were calculated based on the absorbance values of treated and untreated cells (21).

RNA extraction and cDNA synthesis

Total RNA was extracted from the treated and control germinal cells using the GENEzol™ TriRNA Pure Kit (Geneaid, Taiwan), according to the manufacturer's instructions. RNA concentration was determined using an Implen Nano Photometer. First-strand cDNA was synthesized from 1 µg of total RNA using AccuPower® RocketScript™ Cycle RT PreMix (Bioneer), according to the manufacturer's instructions using oligo (dT)20 and random hexamer primers.

Gene expression analysis

The relative expression of the target genes HSP60, HSP70-5 and HSP90-5 was investigated using the reverse transcription quantitative polymerase chain reaction (RT-qPCR) technique. Gene-specific primers were designed to target mRNA and used for amplification of the target genes. The HSP60 transcript was amplified using HSP60_F (5'-CCAGGATGCCTGTGTTCTT-3') and HSP60_R (5'-CCTCAGCAACAATGACGAGA-3'), producing a 113-bp amplicon. The HSP70-5 transcript was amplified using HSP70_5F (5'-AAGGGAGGTATTGTTGGGATGG-3') and HSP70_5R (5'-CCACTCCACCGTCTGTAGGT-3'), producing a 179-bp amplicon. The HSP90-5 transcript was amplified using HSP90_5F (5'-AGCTCTGCACCCACTGATCT-3') and HSP90_5R (5'-GGATCCTTTGCGATTTTGAA-3'), producing a 155-bp amplicon. Actin-β was used as the reference gene and was amplified using Actin-2_F (5'-CGAGCAGGAAATGATAACG-3') and Actin-2_R (5'-GAACAGGGCTTCAGGACAC-3'), producing a 118-bp amplicon. Each qPCR reaction was performed in a final volume of 20 µL, consisting of 10 µL of GoTaq® qPCR Master Mix, 0.1 µM of each primer and 5 µL of fivefold diluted cDNA. The amplification was performed under the following conditions: an initial denaturation at 95°C for 2 min, followed by 50 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Primer efficiency and amplification specificity were assessed using qPCR and melting-curve analysis. The relative expression levels of the target genes were normalized to Actin-2 expression and calculated using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Statistical analyses were conducted using SPSS Statistics version 22. The Chi-square test, independent-samples t-test and one-way and two-way analyses of variance (ANOVA) were applied, as appropriate to assess differences among the experimental groups. Statistical significance was set at $p < 0.05$.

RESULTS

Detection of HSP Genes in hydatids and germinal cells

PCR amplification confirmed the presence of the HSP60, HSP70-5 and HSP90-5 genes in both the germinal cells and protoscolices obtained from hydatid cysts isolated from naturally infected sheep livers. Moreover, to verify the conservation and genetic identity of the amplified HSP genes, PCR products of each gene were randomly selected for bidirectional sanger sequencing using the specific primers designed in the present study. The obtained forward and reverse sequences were assembled and subsequently aligned with the corresponding reference sequences of the *E. granulosus* G1 strain. The findings showed that sequence alignment of HSP90-5 revealed 100% identity with its corresponding G1 reference sequence (Figure 1). Like HSP90-5, HSP70-5 and HSP60 had 100% identity with their corresponding G1 reference sequences (Figures 2, 3). Thus, the complete sequence concordance observed for all three genes confirms their high nucleotide conservation in the examined local *E. granulosus* G1 isolates.

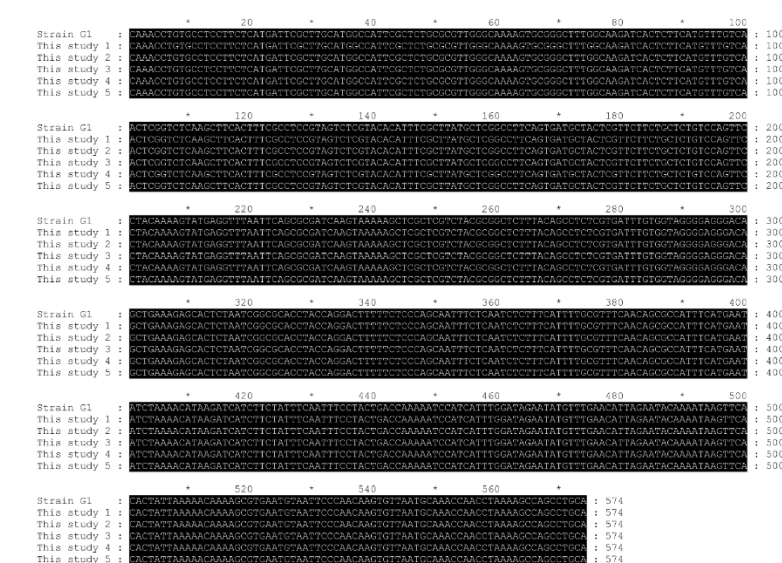


Figure 1. An alignment of the HSP90-5 sequences in the current study with the reference G1 strain sequence.

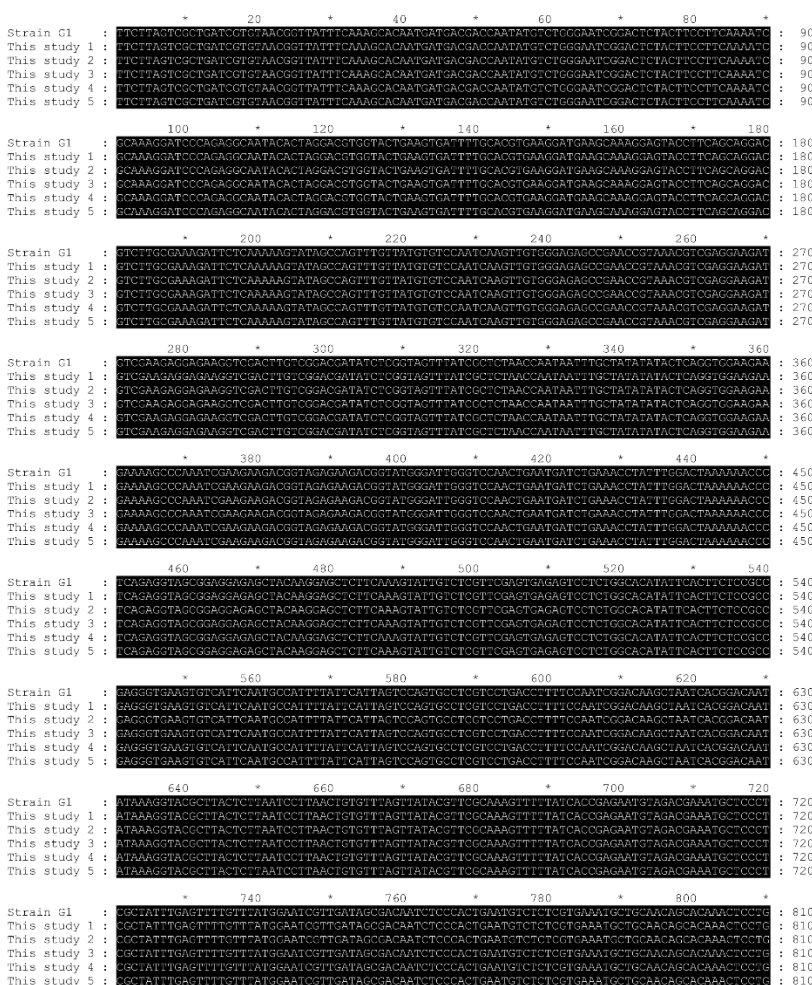


Figure 2. An alignment of the HSP70-5 sequences in the current study with the reference G1 strain sequence.

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      *           20           *           40           *           60           *           80
Strain G1 : TCTCGTCATTGTTGCTGAGGATGTTGAGGGAGAAGCGCTTACTGCCCTCGTTCTCAACCGTTTGAAGCTTGGTCTCCAAGTTTGC : 85
This study 1 : TCTCGTCATTGTTGCTGAGGATGTTGAGGGAGAAGCGCTTACTGCCCTCGTTCTCAACCGTTTGAAGCTTGGTCTCCAAGTTTGC : 85
This study 2 : TCTCGTCATTGTTGCTGAGGATGTTGAGGGAGAAGCGCTTACTGCCCTCGTTCTCAACCGTTTGAAGCTTGGTCTCCAAGTTTGC : 85
This study 3 : TCTCGTCATTGTTGCTGAGGATGTTGAGGGAGAAGCGCTTACTGCCCTCGTTCTCAACCGTTTGAAGCTTGGTCTCCAAGTTTGC : 85
This study 4 : TCTCGTCATTGTTGCTGAGGATGTTGAGGGAGAAGCGCTTACTGCCCTCGTTCTCAACCGTTTGAAGCTTGGTCTCCAAGTTTGC : 85
This study 5 : TCTCGTCATTGTTGCTGAGGATGTTGAGGGAGAAGCGCTTACTGCCCTCGTTCTCAACCGTTTGAAGCTTGGTCTCCAAGTTTGC : 85

      *           100          *           120          *           140          *           160          *
Strain G1 : GCCGTCAAATCACCTGGGTTTGGGGACAATCGCAAGAATACGCTACGTGATATGGCTATTGCAACCGGTGGTGTCTAGGTTACT : 170
This study 1 : GCCGTCAAATCACCTGGGTTTGGGGACAATCGCAAGAATACGCTACGTGATATGGCTATTGCAACCGGTGGTGTCTAGGTTACT : 170
This study 2 : GCCGTCAAATCACCTGGGTTTGGGGACAATCGCAAGAATACGCTACGTGATATGGCTATTGCAACCGGTGGTGTCTAGGTTACT : 170
This study 3 : GCCGTCAAATCACCTGGGTTTGGGGACAATCGCAAGAATACGCTACGTGATATGGCTATTGCAACCGGTGGTGTCTAGGTTACT : 170
This study 4 : GCCGTCAAATCACCTGGGTTTGGGGACAATCGCAAGAATACGCTACGTGATATGGCTATTGCAACCGGTGGTGTCTAGGTTACT : 170
This study 5 : GCCGTCAAATCACCTGGGTTTGGGGACAATCGCAAGAATACGCTACGTGATATGGCTATTGCAACCGGTGGTGTCTAGGTTACT : 170

      180          *           200          *           220          *           240          *
Strain G1 : TGTCCCTGTTTACCCTGCGCTCTAGGTGTTTGGTGATGAGGCGGATATGTACAACTTGAGGATGCCAGCTCCAAGATCTGGGT : 255
This study 1 : TGTCCCTGTTTACCCTGCGCTCTAGGTGTTTGGTGATGAGGCGGATATGTACAACTTGAGGATGCCAGCTCCAAGATCTGGGT : 255
This study 2 : TGTCCCTGTTTACCCTGCGCTCTAGGTGTTTGGTGATGAGGCGGATATGTACAACTTGAGGATGCCAGCTCCAAGATCTGGGT : 255
This study 3 : TGTCCCTGTTTACCCTGCGCTCTAGGTGTTTGGTGATGAGGCGGATATGTACAACTTGAGGATGCCAGCTCCAAGATCTGGGT : 255
This study 4 : TGTCCCTGTTTACCCTGCGCTCTAGGTGTTTGGTGATGAGGCGGATATGTACAACTTGAGGATGCCAGCTCCAAGATCTGGGT : 255
This study 5 : TGTCCCTGTTTACCCTGCGCTCTAGGTGTTTGGTGATGAGGCGGATATGTACAACTTGAGGATGCCAGCTCCAAGATCTGGGT : 255

      260          *           280          *           300          *           320          *           340
Strain G1 : AGAGTTGGTGAGGTTGTTATCACCAAGGACGACACTCTTTATGCGCGGCCACGGTAATAAAGCCGATGTTGACAAGCGCATTT : 340
This study 1 : AGAGTTGGTGAGGTTGTTATCACCAAGGACGACACTCTTTATGCGCGGCCACGGTAATAAAGCCGATGTTGACAAGCGCATTT : 340
This study 2 : AGAGTTGGTGAGGTTGTTATCACCAAGGACGACACTCTTTATGCGCGGCCACGGTAATAAAGCCGATGTTGACAAGCGCATTT : 340
This study 3 : AGAGTTGGTGAGGTTGTTATCACCAAGGACGACACTCTTTATGCGCGGCCACGGTAATAAAGCCGATGTTGACAAGCGCATTT : 340
This study 4 : AGAGTTGGTGAGGTTGTTATCACCAAGGACGACACTCTTTATGCGCGGCCACGGTAATAAAGCCGATGTTGACAAGCGCATTT : 340
This study 5 : AGAGTTGGTGAGGTTGTTATCACCAAGGACGACACTCTTTATGCGCGGCCACGGTAATAAAGCCGATGTTGACAAGCGCATTT : 340

      *           360          *           380          *           400          *           420
Strain G1 : CTCAACTTCGTGATGAGATTAGCGCTAGCAGTAGCGATTATGAAAGGAAAAAATGCAAGAACGCTTTCGAAAACCTTCCAACGG : 425
This study 1 : CTCAACTTCGTGATGAGATTAGCGCTAGCAGTAGCGATTATGAAAGGAAAAAATGCAAGAACGCTTTCGAAAACCTTCCAACGG : 425
This study 2 : CTCAACTTCGTGATGAGATTAGCGCTAGCAGTAGCGATTATGAAAGGAAAAAATGCAAGAACGCTTTCGAAAACCTTCCAACGG : 425
This study 3 : CTCAACTTCGTGATGAGATTAGCGCTAGCAGTAGCGATTATGAAAGGAAAAAATGCAAGAACGCTTTCGAAAACCTTCCAACGG : 425
This study 4 : CTCAACTTCGTGATGAGATTAGCGCTAGCAGTAGCGATTATGAAAGGAAAAAATGCAAGAACGCTTTCGAAAACCTTCCAACGG : 425
This study 5 : CTCAACTTCGTGATGAGATTAGCGCTAGCAGTAGCGATTATGAAAGGAAAAAATGCAAGAACGCTTTCGAAAACCTTCCAACGG : 425

      *           440          *           460          *           480          *           500          *
Strain G1 : TGTTCGCGTCATTAAAGTTGGTGAAGTAGCGAGGTAGAGGTTTCGGAGAAAAAGGATCGCTACACGGATGCCTTGAACTCGACC : 510
This study 1 : TGTTCGCGTCATTAAAGTTGGTGAAGTAGCGAGGTAGAGGTTTCGGAGAAAAAGGATCGCTACACGGATGCCTTGAACTCGACC : 510
This study 2 : TGTTCGCGTCATTAAAGTTGGTGAAGTAGCGAGGTAGAGGTTTCGGAGAAAAAGGATCGCTACACGGATGCCTTGAACTCGACC : 510
This study 3 : TGTTCGCGTCATTAAAGTTGGTGAAGTAGCGAGGTAGAGGTTTCGGAGAAAAAGGATCGCTACACGGATGCCTTGAACTCGACC : 510
This study 4 : TGTTCGCGTCATTAAAGTTGGTGAAGTAGCGAGGTAGAGGTTTCGGAGAAAAAGGATCGCTACACGGATGCCTTGAACTCGACC : 510
This study 5 : TGTTCGCGTCATTAAAGTTGGTGAAGTAGCGAGGTAGAGGTTTCGGAGAAAAAGGATCGCTACACGGATGCCTTGAACTCGACC : 510

      520          *           540          *           560          *           580          *
Strain G1 : CGTGCTGCTGTTGAAGAGGGCATTGTTCTGGAGGTGGCGTGCTTTGTTGCGGTGATTGAGGTTCTTGACAGGATTGAAGGCA : 595
This study 1 : CGTGCTGCTGTTGAAGAGGGCATTGTTCTGGAGGTGGCGTGCTTTGTTGCGGTGATTGAGGTTCTTGACAGGATTGAAGGCA : 595
This study 2 : CGTGCTGCTGTTGAAGAGGGCATTGTTCTGGAGGTGGCGTGCTTTGTTGCGGTGATTGAGGTTCTTGACAGGATTGAAGGCA : 595
This study 3 : CGTGCTGCTGTTGAAGAGGGCATTGTTCTGGAGGTGGCGTGCTTTGTTGCGGTGATTGAGGTTCTTGACAGGATTGAAGGCA : 595
This study 4 : CGTGCTGCTGTTGAAGAGGGCATTGTTCTGGAGGTGGCGTGCTTTGTTGCGGTGATTGAGGTTCTTGACAGGATTGAAGGCA : 595
This study 5 : CGTGCTGCTGTTGAAGAGGGCATTGTTCTGGAGGTGGCGTGCTTTGTTGCGGTGATTGAGGTTCTTGACAGGATTGAAGGCA : 595

      600          *           620          *           640          *           660          *           680
Strain G1 : AGAACAATGATCAACAAATGGTTAGTTAGTGACTTTTCTTTTGGTTATGTGTTTCACTTTGCGCTTTGGCACCCTGGTTTACTGTT : 680
This study 1 : AGAACAATGATCAACAAATGGTTAGTTAGTGACTTTTCTTTTGGTTATGTGTTTCACTTTGCGCTTTGGCACCCTGGTTTACTGTT : 680
This study 2 : AGAACAATGATCAACAAATGGTTAGTTAGTGACTTTTCTTTTGGTTATGTGTTTCACTTTGCGCTTTGGCACCCTGGTTTACTGTT : 680
This study 3 : AGAACAATGATCAACAAATGGTTAGTTAGTGACTTTTCTTTTGGTTATGTGTTTCACTTTGCGCTTTGGCACCCTGGTTTACTGTT : 680
This study 4 : AGAACAATGATCAACAAATGGTTAGTTAGTGACTTTTCTTTTGGTTATGTGTTTCACTTTGCGCTTTGGCACCCTGGTTTACTGTT : 680
This study 5 : AGAACAATGATCAACAAATGGTTAGTTAGTGACTTTTCTTTTGGTTATGTGTTTCACTTTGCGCTTTGGCACCCTGGTTTACTGTT : 680

      *           700          *           720
Strain G1 : GTAGGCATCGACATTGTGAAGAAAGCCCTCGAAACTCCCGCTTACAC : 727
This study 1 : GTAGGCATCGACATTGTGAAGAAAGCCCTCGAAACTCCCGCTTACAC : 727
This study 2 : GTAGGCATCGACATTGTGAAGAAAGCCCTCGAAACTCCCGCTTACAC : 727
This study 3 : GTAGGCATCGACATTGTGAAGAAAGCCCTCGAAACTCCCGCTTACAC : 727
This study 4 : GTAGGCATCGACATTGTGAAGAAAGCCCTCGAAACTCCCGCTTACAC : 727
This study 5 : GTAGGCATCGACATTGTGAAGAAAGCCCTCGAAACTCCCGCTTACAC : 727
    
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Figure 3. An alignment of the HSP60 sequences in the current study with the reference G1 strain sequence.

Effect of H2O2 on cell proliferation

The cytotoxicity assay demonstrated a concentration-dependent inhibitory effect of H₂O₂ on G1 strain germinal cells. The lowest inhibition rate was observed at 10 µM H₂O₂ (14.11%), whereas the highest inhibition (62.16%) occurred at 500 µM. In addition, the statistical analysis revealed significant differences in cell inhibition among the tested H₂O₂ concentrations. Furthermore, the inhibitory effect progressively increased with increasing H₂O₂ concentration, indicating a clear positive relationship between exposure concentration and cytotoxicity. The half-maximal inhibitory concentration was estimated at 350 µM, (Figure 4).

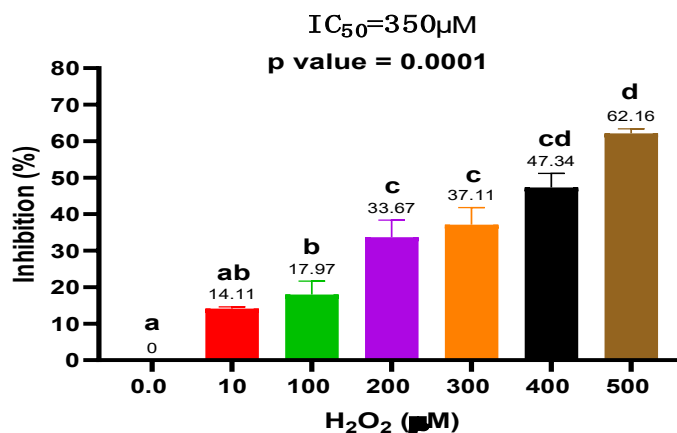


Figure 4. Inhibitory effect of H₂O₂ at various exposure concentrations (0 - 500 μM). Data are presented as mean ± SE. Values with different letters (a–d) indicate statistically significant differences between concentrations. The half-maximal inhibitory concentration (IC₅₀) was determined to be 350 μM.

Effect of H₂O₂ on the expression of HSP genes

Exposure of cultured G1 cells to 200 μM H₂O₂ and the half inhibitory concentration (350 μM) resulted in increased expression of HSP90-5, HSP70-5 and HSP60 compared with the control group. The observed differences were statistically significant at $P \leq 0.05$.

Effect of H₂O₂ on the expression of HSP 5-90 gene

Exposure of germinal cells to H₂O₂ at 200 μM and at the IC₅₀ concentration (350 μM) resulted in an increase in the expression of the HSP90-5 gene, which encodes a secretory protein, compared with the control group. At 200 μM H₂O₂, the mean expression level of HSP90-5 increased to 3.21-fold relative to the control group. Exposure to the IC₅₀ concentration (350 μM) also resulted in an increase in HSP90-5 expression, exceeding three-fold compared with the control group. These findings indicate that H₂O₂ exposure induced an elevated transcriptional response of HSP90-5 in the treated cells. Statistical analysis showed significant differences in HSP90-5 expression between the H₂O₂-treated groups at 200 and 350 μM and the control group at $P = 0.05$, (Figure 5).

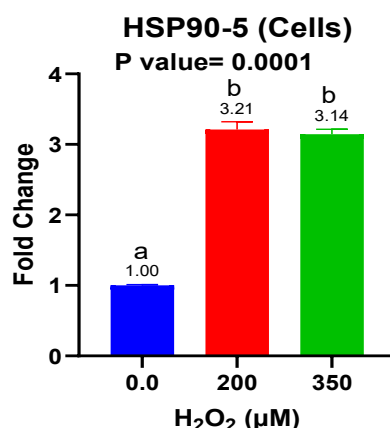


Figure 5. Relative mRNA expression fold change of HSP90-5 in response to H₂O₂ exposure. Bars display the fold change values (1.00, 3.21, and 3.14) at 0.0, 200, and 350 μM H₂O₂. Different letters (a, b) above the bars indicate significant differences between treatments ($P < 0.05$).

Effect of H₂O₂ on the expression of HSP 75-0 gene

Exposure of germinal cells to H₂O₂ at 200 μ M and at the IC₅₀ concentration (350 μ M) markedly increased the expression of the HSP70-5 gene, which encodes a cytoplasmic protein, compared with the control group. At 200 μ M H₂O₂, the mean expression level of HSP70-5 increased to 187.56-fold relative to the control group. At the IC₅₀ concentration of 350 μ M, the expression level increased further, reaching 238.92-fold compared with the control group. Thus, HSP70-5 exhibited a substantially greater transcriptional response than that observed for the other investigated HSP genes. Statistical analysis revealed significant differences in HSP70-5 expression between both H₂O₂-treated groups (200 and 350 μ M) and the control group at $P = 0.05$, (Figure 6).

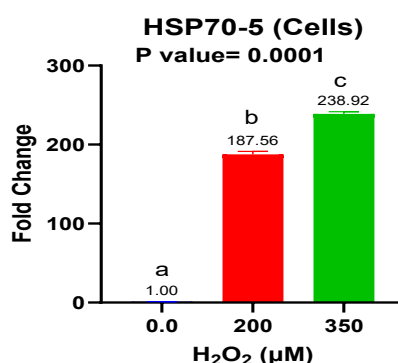


Figure 6. Relative mRNA expression fold change of HSP70-5 in response to H₂O₂ exposure. Bars display the fold change values (1.00, 187.56 and 238.92) at 0.0, 200, and 350 μ M H₂O₂.

Different letters (a, b) above the bars indicate significant differences between treatments ($P < 0.05$).

Effect of H₂O₂ on the expression of HSP 60 gene

Exposure of germinal cells to H₂O₂ at 200 μ M and at the IC₅₀ concentration (350 μ M) increased the expression of the HSP60 gene, which encodes a mitochondrial protein, relative to the control group. At 200 μ M H₂O₂, the mean expression level of HSP60 increased to 2.51-fold compared with the control group. At 350 μ M H₂O₂, the expression level increased to 3.08-fold relative to the control group. Therefore, the expression of HSP60 was higher at the IC₅₀ concentration than at 200 μ M. Statistical analysis demonstrated significant differences in HSP60 expression between both H₂O₂-treated groups (200 and 350 μ M) and the control group at $P = 0.05$, (Figure 7).

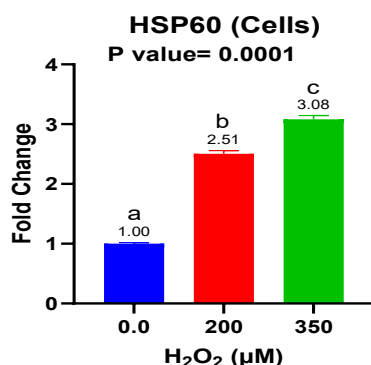


Figure 7. Relative mRNA expression fold change of HSP60 in response to H₂O₂ exposure. Bars display the fold change values (1.00, 2.51 and 3.08) at 0.0, 200, and 350 μ M H₂O₂. Different letters (a, b) above the bars indicate significant differences between treatments ($P < 0.05$).

Comparative Expression of HSP Genes under H₂O₂-Induced Stress

The relative mRNA expression profiles of three heat shock protein genes (HSP90-5, HSP70-5, and HSP60) were analyzed in germinal cells subjected to oxidative stress mediated by H₂O₂ at sub-inhibitory (200 μ M) and IC₅₀ (350 μ M) concentrations. As summarized in (Figure 8), exposure to H₂O₂ triggered a statistically significant upregulation in all target genes relative to the untreated control ($P < 0.05$), indicative of a robust activation of the cellular heat shock response (HSR) machinery. Despite the universal induction, the transcriptional magnitude differed profoundly across the gene families. The cytoplasmic chaperone gene HSP70-5 exhibited a hyper-inducible phenotype, with transcript levels escalating dramatically to 187.56-fold and 238.92-fold following treatment with 200 μ M and 350 μ M H₂O₂, respectively. Conversely, the secretory HSP90-5 and mitochondrial HSP60 genes displayed a more restrained upregulation. Expression of HSP90-5 rose to 3.21-fold and 3.92-fold, whereas HSP60 increased to 2.51-fold and 3.08-fold at the corresponding concentrations. Furthermore, a clear dose-dependent relationship was evident, wherein the IC₅₀ concentration consistently provoked a stronger transcriptional response than the 200 μ M treatment, reflecting a graded defensive allocation commensurate with the intensity of oxidative damage.

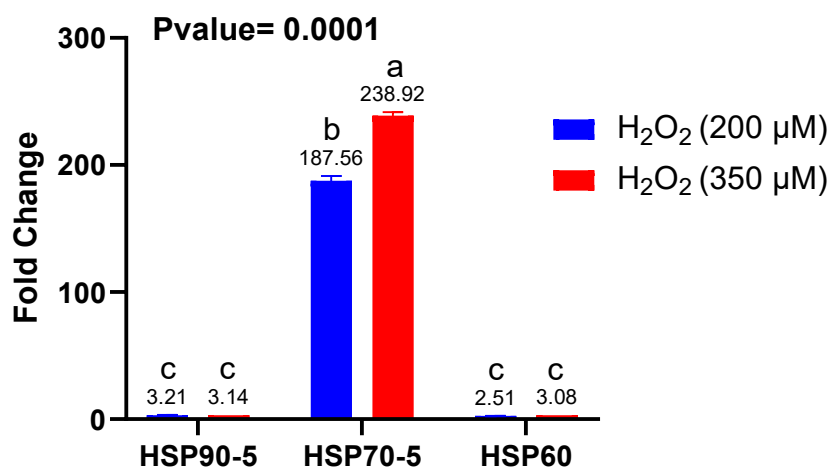


Figure 8. Relative mRNA expression fold change of HSPs in response to H₂O₂ exposure. Bars display the fold change values at 200, and 350 μ M H₂O₂. Different letters above the bars indicate significant differences between treatments ($P < 0.05$).

DISCUSSION

The findings of the present study, when considered collectively, provide converging molecular, physiological, and functional evidence regarding the role of heat shock proteins in the biology of *E. granulosus* G1, and offer several avenues that merit further exploration in future work. Sequence analysis of HSP60, HSP70-5 and HSP90-5 revealed complete nucleotide identity between the amplified regions of these genes and their corresponding G1 reference sequences with no substitutions, insertions, or deletions detected in any of the three loci. Such a high degree of conservation is not unexpected given the essential and evolutionarily constrained functions that HSPs perform in protein folding and cellular homeostasis, and it aligns with earlier reports describing conservation of HSP90 sequences across *E. granulosus sensu stricto* genotypes as a potential basis for species- and genotype-level identification (22), as well as with the improved resolution offered by the chromosome-scale G1 reference genome (23). Taken together with previous work employing mitochondrial markers to confirm G1-strain identity in Iraqi isolates (18,19), the present results support the use of these three HSP loci as complementary molecular markers for confirming G1-strain identity, while acknowledging that this conclusion applies specifically to the amplified regions examined and cannot be extrapolated to genome-wide identity between the local isolates and the reference strain. The cytotoxicity assay demonstrated a clear, concentration-dependent reduction in the viability of G1 germinal cells following H₂O₂ exposure, with an estimated IC₅₀ of 350 μ M. What makes this finding particularly noteworthy is the antioxidant background against which it occurred. Unlike many eukaryotic organisms, *E. granulosus* lacks detectable catalase and glutathione peroxidase activity and depends almost

entirely on a single enzymatic route, the thioredoxin peroxidase system (EgTPx) for the detoxification of hydrogen peroxide (12). Despite this comparatively narrow enzymatic defense, the tolerance exhibited by G1 germinal cells in the present study was substantial, and indeed exceeded that reported for normal human cells under comparable experimental conditions: normal human pulmonary fibroblasts, for instance, show an IC_{50} of approximately 50 μM when exposed to H_2O_2 for 24 hours (10), roughly seven-fold lower than the concentration tolerated by the parasite's germinal cells in this study. This comparison suggests that *E. granulosus* has evolved compensatory mechanisms that allow it to withstand oxidative insult more effectively than mammalian somatic cells, despite possessing a more limited enzymatic antioxidant arsenal. The gene-expression data provide a plausible explanation for this apparent paradox. Exposure of G1 germinal cells to H_2O_2 significantly up-regulated all three HSP genes at both sub-inhibitory (200 μM) and IC_{50} (350 μM) concentrations indicating that the parasite actively mobilizes its chaperone machinery in response to oxidative challenge rather than relying solely on peroxide-scavenging enzymes. The magnitude of this response, however, differed considerably among the three genes. HSP60, a mitochondrial chaperone, and HSP90-5, a secretory chaperone, both showed modest and concentration-dependent increases consistent with their established supporting roles in protein folding and stabilization (24,25,26). By contrast, the cytoplasmic chaperone HSP70-5 exhibited a markedly disproportionate response, reaching 238.92-fold at the IC_{50} concentration far surpassing the induction levels previously reported for HSP70 in proteomic studies of H_2O_2 -exposed *E. granulosus* (14). This pattern indicates that the parasite effectively compensates for its restricted enzymatic antioxidant capacity by heavily deploying HSP70-5 to safeguard the cellular proteome from the downstream consequences of oxidative damage, rather than by neutralizing reactive oxygen species directly. This interpretation is consistent with the wider genomic evidence for an expanded and potentially functionally diversified HSP70 gene family in tapeworms (27). The involvement of HSP70 in the parasite's biology also carries implications beyond basic stress physiology. HSP70 antigens of *E. granulosus* are known to be immunologically reactive, with IgG, IgG4, and IgE antibodies detected in the sera of patients with cystic echinococcosis (15), and this immunoreactivity has already been translated into a practical diagnostic application in the form of a rapid colloidal-gold immunochromatographic strip for field detection of the parasite in sheep (16). Considered alongside the sequence-based diagnostic potential of the conserved HSP loci identified in the present study, these observations reinforce the view that HSP70-5, together with the other conserved HSP genes, could serve a dual purpose: as a molecular marker supporting strain identification, and as a serological or immunodiagnostic indicator of active infection. In related helminth systems, HSP70 orthologues have further been shown to elicit protective immune responses; Ts-Hsp70 of *Trichinella spiralis*, for example, activates dendritic cells via TLR2 and TLR4 signaling and confers partial protective immunity in experimental infection (17), a precedent that lends support to the candidacy of HSP70-5 as a potential vaccine target in *E. granulosus*. Taken together, the present findings depict a parasite that, despite a comparatively narrow enzymatic antioxidant defense, achieves a level of oxidative-stress tolerance exceeding that of normal mammalian cells through the coordinated and disproportionately strong mobilization of molecular chaperones most notably HSP70-5. This chaperone-based strategy, layered upon the parasite's antioxidant machinery, may constitute a key mechanism underlying the prolonged survival of *E. granulosus* within the hostile, ROS-rich environment of the intermediate host (28,29). Given the genetic conservation, immunodiagnostic relevance, and immunostimulatory precedents associated with HSP70 in related parasites, future research should place greater emphasis on characterizing these genes in *E. granulosus*, both as candidate markers for the serological detection of infection and as potential antigenic targets in vaccine development.

CONCLUSION

The present study demonstrated that HSP60, HSP70-5 and HSP90-5 were consistently detected in all examined hydatid cyst samples and showed 100% nucleotide identity with their corresponding G1 reference sequences, confirming their high genetic conservation and supporting their potential use as reliable molecular markers for diagnosing the *E. granulosus* G1 strain. Building on this conserved genetic background, the cytotoxicity results revealed that, despite the parasite's lack of the major enzymatic antioxidant defenses conventionally relied upon by many organisms, G1 germinal cells nevertheless exhibited a notably high tolerance to H_2O_2 induced oxidative stress, withstanding concentrations up to an IC_{50} of 350 μM before viability was substantially compromised. This unexpected resilience is explained by the gene-expression findings, which showed that the parasite possesses a remarkable capacity to mobilize and up-regulate its HSP genes in order to counteract oxidative injury, with HSP70-5 in particular displaying an exceptionally strong transcriptional response that far exceeded the more moderate induction observed for HSP60 and HSP90-5. Taken together, these findings suggest that the coordinated and highly conserved HSP gene

network and the parasite's evident ability to deploy it efficiently under stress may represent the underlying molecular secret enabling *E. granulosus* to survive and persist within intermediate hosts for prolonged periods. In addition, H₂O₂ produced a concentration-dependent cytotoxic effect on G1 germinal cells, with an IC₅₀ of 350 µM. Taken together, the findings suggest that the conserved HSP genes contribute to the ability of *E. granulosus* G1 germinal cells to tolerate oxidative stress, although this protective response has a physiological limit. HSP70-5, in particular, may therefore represent a promising molecular target for future investigations aimed at understanding parasite survival and identifying novel therapeutic strategies against cystic echinococcosis.

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Conflict of interest

The authors declare that no competing interests exist regarding the publication of this study, and no financial support or funding was received for the preparation or publication of the manuscript.

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