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# Malarial pigment enhances heat shock protein-27 in THP-1 cells: new perspectives for *in vitro* studies on monocyte apoptosis prevention

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ABSTRACT

**Objective:** To investigate the effect of malarial pigment (hemozoin, HZ) on expression of heat shock proteins (HSPs) and cell viability in human monocytes by using a stable cell line (THP-1 cells). **Methods:** THP-1 cells were fed with native HZ or treated with pro-apoptotic molecule gliotoxin for 9 h. Thereafter, the protein expression of HSP-27 and HSP-70 was evaluated by western blotting. Alternatively, HZ-fed cells were cultured up to 72 h and cell viability parameters (survival, apoptosis and necrosis rates) were measured by flow cytometric analysis. **Results:** HZ increased basal protein levels of HSP-27 without altering those of HSP-70 in THP-1 cells, and promoted long-term cell survival without inducing apoptosis. As expected, gliotoxin inhibited HSP-27 protein expression and promoted long-term cell apoptosis. **Conclusions:** Present data show that HZ prevents cell apoptosis and enhances the expression of anti-apoptotic HSP-27 in THP-1 cells, confirming the previous evidences obtained from HZ-fed immunopurified monocytes. Since the use of a stable cell line is pivotal to perform HSP-27 silencing experiments, monocytic THP-1 cells could be a good candidate line for such an approach, which is heavily required to clarify the role of HSP-27 in survival of impaired HZ-fed monocytes during falciparum malaria.

## 1. Introduction

Despite the recent successes obtained by the new Malaria Eradication Program, started in 2007 by the Bill and Melinda Gates Foundation and rapidly endorsed by the World Health Organization and the Roll Back Malaria association, 2.37 billion people are still at risk of infection by *Plasmodium falciparum (P. falciparum)*, the intraerythrocytic parasite responsible for the most serious form of malaria[1]. Phagocytes such as monocytes, the versatile cells that act as scavengers to rid the body of apoptotic and senescent cells and other debris, avidly phagocytose parasitized red blood cells[2], but are not able to destroy hemozoin (HZ), the ferriprotoporphyrin IX crystal derived from undigested host haemoglobin heme, present in late stages of parasitized red blood cells (trophozoites) and in residual bodies shed after schizogony[3]. As a consequence, undigested HZ, which persists for at least 72 hours in the otherwise intact lysosomes of human monocytes[4], impairs a large number of their functions, such as repeated phagocytosis[4], antigen presentation[5], oxidative burst[6], bacterial killing[7], and coordination of erythropoiesis[8]. Additionally, HZ-fed monocytes have been shown to produce large amounts of cytokines, including tumor necrosis factor alpha (TNF alpha)[9-12] and interleukin-1beta (IL-1beta)[12-14], and several chemokines, such as interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), monocyte inflammatory protein-1alpha (MIP-1alpha) and MIP-1beta[14], and to enhance expression, release and activity of cytokine-dependent enzymes matrix metalloproteinase-9 (MMP-9)[9, 12-14] and lysozyme[10]. Nevertheless, histology of autopsy tissues from patients with severe malaria shows the abundant presence of HZ-laden Kupffer cells and other tissue macrophages[15, 16], indicating that their defects in function and cytokine/enzyme production are not sufficient to induce apoptosis and death. In a previous work, it was shown that human monocytes immunopurified from peripheral blood and loaded with native pure HZ did not undergo apoptosis up to 72 hours, while protein levels of anti-apoptotic heat shock protein-27 (HSP-27) in HZ-fed cell lysates were higher than those of control cells[14]. However, a direct causal relationship between HSP-27 expression and survival of HZ-fed monocytes has not yet been demonstrated. To reach this goal, a gene silencing approach (RNA interference) might be useful[17], but using a stable cell line instead of immunopurified cells should be more indicated in order to perform such an approach.

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For this reason, the previous experiments performed on immunopurified monocytes were repeated here on THP-1 cells, a stable and easy-to-handle human monocytic cell line[18], which matures into macrophage-like adherent cells following stimulation with phorbol 12-myristate 13-acetate[19] or 1alpha, 25-dihydroxy vitamin D3[20]. In present work, THP-1 cells were able to phagocytose HZ *in vitro* with efficiency comparable to immunopurified cells. Results showed that in THP-1 cells HZ enhanced HSP-27 (but not HSP-70) protein expression and prevented apoptosis, confirming previous evidences obtained from immunopurified monocytes. Consequently, THP-1 cells are good candidates for future HSP-27 silencing experiments aimed to understand the role of HSP-27 in the detrimental survival of HZ-fed monocytes during malaria.

## 2. Materials and methods

### 2.1. Materials

Unless otherwise stated, reagents were obtained from Sigma-Aldrich, St. Louis, MO. Sterile plastics were from Costar, Cambridge, UK; Panserin 601 monocyte medium was from PAN Biotech, Aidenbach, Germany; Percoll was from Pharmacia, Uppsala, Sweden; Diff-Quik parasite stain was from Baxter Dade AG, Dudingen, Switzerland; ECL Kit, HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were from GE-Healthcare, Milan, Italy; Geldoc computerized densitometer and electrophoresis reagents were from Bio-Rad Laboratories, Hercules, CA; TACS Annexin Kit for apoptosis detection by flow cytometry was from Trevigen, Gaithersburg, MD; FACSCalibur cytometer and Cell Quest software were from BD Biosciences, San Jose, CA; bicinchoninic acid protein assay was from Pierce, Rockford, IL; anti-HSP-27 polyclonal Ab was from Stressgen, Ann Arbor, MI.

### 2.2. Culturing of P. falciparum and isolation of HZ

*P. falciparum* parasites (Palo Alto strain, mycoplasma-free) were kept in culture as described[9]. After centrifugation at 5 000 × g on a discontinuous Percoll-mannitol density gradient, HZ was collected from the 0%-40% interphase, washed five times with 10 mM HEPES (pH 8.0) containing 10 mM mannitol at 4 ℃ and once with PBS, and stored at 20% (v/v) in PBS at -20 ℃ or immediately used for opsonization and phagocytosis.

### 2.3. Culturing of THP-1 cells

The human monocyte cell line THP-1 was grown in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-Glutamine, 100 U/mL penicillin and 100 $\mu$g/mL streptomycin, by the replacement of fresh medium twice a week. For general maintenance, the cells were seeded at $5\times10^5$. For phagocytosis assay THP-1 cells were plated in 6-well plates ($5\times10^5$/well) in Panserin medium.

### 2.4. Phagocytosis of opsonized HZ and treatment with gliotoxin

HZ washed once and finely dispersed at 30% (v/v) in PBS was added to the same volume of fresh human AB serum (AVIS blood bank) and incubated for 30 min at 37 ℃ as described[9]. Phagocytosis was started by adding to THP-1 cells opsonized HZ (50 RBC equivalents, in terms of heme content, per monocyte). The plates were then incubated in Panserin 601 medium in a humidified $CO_2$/air-incubator at 37 ℃ for the indicated times (9 h for HSPs protein expression analysis; 72 h for viability studies). Alternatively, unfed THP-1 cells were incubated with 10 $\mu$M gliotoxin for 9 h.

### 2.5. Anti-HSP-27 and anti-HSP-70 western blotting

Unstimulated, HZ-fed and gliotoxin-treated THP-1 cells were incubated with Panserin 601 monocyte medium in a humidified $CO_2$/air-incubator at 37 ℃ for 9 h. Subsequently cells were washed and lysed at 4 ℃ in lysis buffer containing: 300 mM NaCl, 50 mM Tris, 1% (v/v) Triton-×100, protease and phosphatase inhibitors: 50 ng/mL pepstatin, 50 ng/mL leupeptin, 10 $\mu$g/mL aprotinin. The protein content of the lysate was measured by the bicinchoninic acid assay. Lysates samples (25 $\mu$g protein/lane) were separated by electrophoresis on 8% and 12% polyacrylamide gels under denaturing and reducing conditions, with addition of Laemmli Buffer (100 mM Tris-HCl, pH 6.8, 2% w/v SDS, 20% v/v glycerol, 4% v/v $\beta$-mercaptoethanol), blotted on a polyvinylidene difluoride membrane, and probed with 1:5 000 polyclonal rabbit anti-HSP-27 and 1:2 000 monoclonal anti-HSP-70 antibodies. After 5 min washes, the blot was incubated for 1 h with a 1:10 00 dilution of anti-rabbit or anti-mouse IgG horseradish-peroxidase-labelled antibody and immunoreactivity was detected with ECL Kit. Bands densitometric analysis was performed using Geldoc computerized densitometer.

### 2.6. Viability analysis by flow cytometry

Unstimulated and HZ-fed THP-1 cells were incubated with Panserin 601 monocyte medium in a humidified $CO_2$/air-incubator at 37 ℃ for 72 h. Alternatively, to obtain a positive control for apoptosis, monocytes were incubated with 10 $\mu$M gliotoxin for 9 h. Thereafter, cell viability was evaluated by flow cytometry using FITC-conjugated Annexin V and propidium iodide staining (TACS Annexin Kit), according to the manufacturer's instructions. Briefly, cells were washed with PBS with $Ca^{2+}$ and then incubated for 15 min at 25 ℃ with 0.025 $\mu$g FITC-conjugated Annexin V and 0.5 $\mu$g propidium iodide before analysis with a FACSCalibur cytometer, using Cell Quest software. Live cells were distinguished from apoptotic or necrotic cells by appropriately gated light scatter characteristics. A total of 30 000 events were collected for each sample. Data analysis was performed with WinMDI software.

### 2.7. Statistical analysis

For each set of experiments, data are showed as means±SD or one representative imagine of three independent experiments. All data were analysed by student's *t* test.

## 3. Results

### 3.1. Monocytic THP-1 cells are able to perform HZ phagocytosis

During the following experiments, THP-1 cells were allowed to phagocytose opsonized HZ (50 RBC equivalents, in terms of heme content, per monocyte) for the indicated times. After the end of the phagocytic period, cells were checked by optical microscopy to verify the HZ ingestion. Figure 1 shows the image of one representative HZ-fed monocyte. Additionally, the amount of HZ phagocytosed by monocytes was quantified by luminescence as described previously[21]; on average, each monocyte ingested HZ equivalent to ~8-10 trophozoites in term of ingested heme.

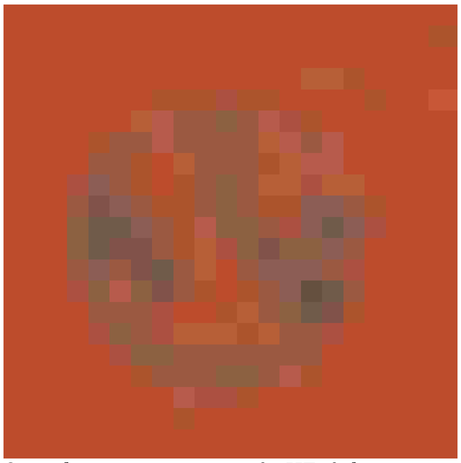

**Figure 1.** Optical microscopy image of a HZ-fed monocyte.

### *3.2. Phagocytosis of HZ enhances HSP-27 protein expression in monocytic THP-1 cells*

Expression of HSP-27 protein was detected in cell lysates by western blotting in unstimulated, HZ-fed and gliotoxin-treated THP-1 cells after 9 h of incubation (Figure 2). The 27 kDa bands in the blot (upper panel, representative image) were analysed by optical densitometry (lower panel, mean values±SD of arbitrary densitometric units from three independent experiments). On average, HSP-27 protein expression increased by 50% in HZ-fed cells versus unstimulated cells, while it was almost totally degraded after gliotoxin treatment. Data were analysed by Student's *t*-test, and following differences were obtained. HZ-fed versus unfed cells: $P<0.02$; gliotoxin-treated versus unfed cells: $P<0.002$; HZ-fed versus gliotoxin-treated cells: $P<0.001$.

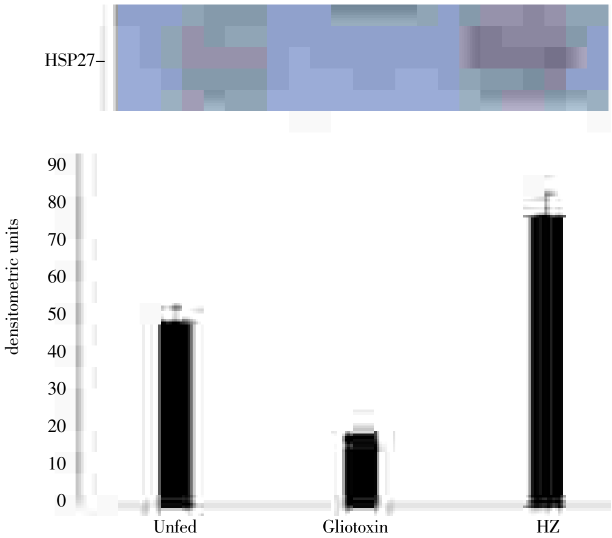


**Figure 2.** HSP-27 protein expression in unstimulated, gliotoxin-treated and HZ-fed monocytic THP-1 cells.

### *3.3. Phagocytosis of HZ does not alter HSP-70 protein expression in monocytic THP-1 cells*

Protein expression of HSP-70 was detected in cell lysates by western blotting in unstimulated, HZ-fed and gliotoxin-treated THP-1 cells after 9 h of incubation (Figure 3). The 70 kDa bands in the blot (upper panel, representative image) were analysed by optical densitometry (lower panel, mean values±SD of arbitrary densitometric units from three independent experiments). Data were analysed by Student's *t*-test and no significant differences among conditions were found.

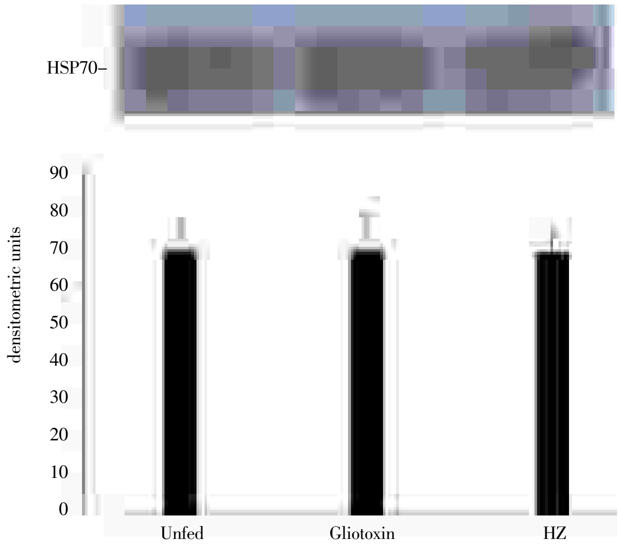


**Figure 3.** HSP-70 protein expression in unstimulated, gliotoxin-treated and HZ-fed monocytic THP-1 cells.

### *3.4. Phagocytosis of HZ prevents apoptosis in monocytic THP-1 cells*

Cell viability was studied by FACS analysis in 72 h incubated unstimulated (Figure 4A), 9 h-gliotoxin-treated (Figure 4B) and 72 h HZ-loaded (Figure 4C) THP-1 cells. Data are expressed as mean percentage±SD of three independent experiments. On average, apoptosis was detected in ~8% unfed cells, in ~4% HZ-fed cells and in 66% gliotoxin-treated cells; necrosis was nearly absent in unfed and HZ-fed cells and ~5% in gliotoxin-treated cells; alive cells were ~92% of total unfed cells, ~95% of total HZ-fed cells and ~29% of total gliotoxin-treated cells. Data were analysed by Student's *t*-test, and following differences were obtained. HZ-fed versus unfed cells: not significant (all parameters: survival, apoptosis, necrosis); HZ-fed versus gliotoxin-treated cells: $P<0.02$ (survival), $P<0.01$ (apoptosis) and not significant (necrosis); unfed versus gliotoxin-treated cells: $P<0.02$ (survival), $P<0.01$ (apoptosis) and not significant (necrosis).

## 4. Discussion

Phagocytosis is a primary function of monocytes, which are versatile cells that act as scavengers to rid the body of apoptotic and senescent cells, and other debris through their phagocytic function. Additionally, monocytes play vital roles in inflammation and repair of damaged tissues, secreting a large number of cytokines, chemokines and growth factors that activate a variety of cell types and recruit them to inflamed tissue compartments. In malaria, circulating monocytes avidly phagocytose HZ, the heme detoxification biocrystal produced by the parasite during hemoglobin catabolism, but the malarial pigment is not degraded, persisting for at least 72 hours in the otherwise intact lysosomes of these cells[4]. As a consequence, numerous monocytes functions are impaired, including repeated phagocytosis[4], antigen presentation[5], oxidative burst[6], bacterial killing[7], and coordination of erythropoiesis[8]. Moreover, phagocytosis of HZ promotes cytokine/chemokine (TNFalpha, IL-1beta, IL-8, MCP-1, MIP-1alpha, MIP-1beta)[9-14] and cytokine-related enzyme (MMP-9, lysozime)[9-14] production. Since monocytes are important in regulating and resolving inflammation, their prolonged survival in tissue compartments could be detrimental and favour progress towards complicated malaria[22]. Histology of autopsy tissues from patients with severe malaria shows the abundant presence of HZ-laden Kupffer cells and other tissue macrophages[15, 16], suggesting that these cells are not able to undergo apoptosis despite their defects in function and cytokine/enzyme production. This hypothesis was confirmed by evidences obtained from a recent work *in vitro*, where we showed by immunocytochemistry and flow cytometry that impaired HZ-fed immunopurified monocytes did not undergo apoptosis up to 72 h[14].

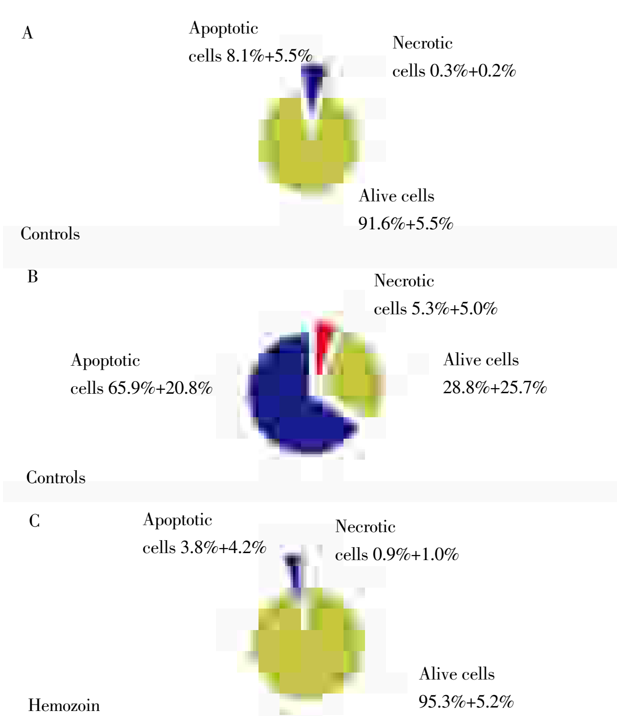


**Figure 4.** Long-term cell viability of unstimulated, gliotoxin-treated and HZ-fed monocytic THP-1 cells.

Monocyte survival can be promoted through several mechanisms, including the NF-kB transcription system and the MAPK cascade, whose major subfamilies are Erk, p38MAPK and JNK[22]. Activation of the NF-kB pathway by HZ has been proposed recently in two models: in human monocytes it has been suggested to be responsible for enhancement of TNF$\alpha$, IL-1$\beta$ and MMP-9 after phagocytosis of native, but not synthetic, HZ[12] while in murine macrophages fed with either native or synthetic HZ (beta-hematin), the activated NF-kB transcription system was related to higher levels of MCP-1, MIP-1$\alpha$ and MIP-1$\beta$ chemokines[23]. Additionally, activation of the Erk1/2 pathway has been described in native and beta-hematin-fed murine macrophages[23, 24], but not yet in humans.

Interestingly, MAPK-mediated induction of antiapoptotic HSP-27 has been reported[25]. The expression of HSPs is induced in response to a wide variety of physiological and environmental insults, these proteins playing an essential role as molecular chaperones able to assist the correct folding of nascent and stress-accumulated misfolded proteins, and to prevent their aggregation. Intracellular HSPs have a protective function, allowing the cells to survive lethal conditions. At the mitochondrial level, HSP-27, through Bid, and HSP-70, by inhibiting Bax, inhibit the mitochondrial release of proapoptotic proteins, while at the postmitochondrial level, HSP-27 binds to cytochrome c, and HSP-70 binds to Apaf1. As a result, the apoptosome formation is inhibited and caspase activation and apoptosis are thereby prevented. HSP-27 can also interact with and inhibit Daxx apoptotic pathway whereas HSP-70 binds to JNK-1, resulting in inhibition of JNK activation[25, 26].

Interestingly, enhanced HSP-27 protein expression has been reported in the previous work performed on non-apoptotic immunopurified monocytes[14]. A major understanding of the mechanisms by which HZ promotes HSP-27 expression and the ensuing apoptosis block is certainly required, in a reasonable perspective to contrast the pathological persistence of circulating impaired monocytes, which might be detrimentally instrumental in inducing the hallmarks of complications of malaria, including cerebral malaria. The best approach to investigate this field should be to perform a HSP-27 silencing on monocytic cells, but an assay system using an established cell line instead of immunopurified monocytes should be more suitable to simplify and standardize laboratory testing[17]. THP-1 cells are a stable and easy-to-handle human monocytic cell line[18], which matures into macrophage-like adherent cells following stimulation with phorbol 12-myristate 13-acetate[19] or 1alpha, 25-dihydroxy vitamin D3[20]. As shown in the results section, THP-1 cells were able to phagocytose HZ *in vitro* with an efficiency comparable to immunopurified cells. Flow cytometric analyses performed at prolonged times (72 h) after phagocytosis of HZ showed that the survival rate of HZ-fed THP-1 cells was similar to that of unfed ones. Additionally, 9 h after the phagocytosis of HZ, THP-1 cells showed enhanced HSP-27 (but not HSP-70) protein expression. As expected, all these results confirmed previous evidences obtained from immunopurified monocytes[14]. Therefore, THP-1 cells are a good cell line candidate in order to perform future HSP-27 silencing experiments, which are certainly warranted in order to understand if HSP-27, usually indicated as a potential target for anticancer drugs, can be also considered as a new target for antimalarial therapies. Indeed, the cross-over between results from antimalarial and anticancer pharmacological research is intriguing. For example, recent evidences showed that quercetin, a natural antioxidant flavonoid able to block the phosphorylation of the I$\kappa$Balpha protein[27], inhibited HSPs expression and caused higher sensitization to doxorubicin in neuroblastoma[28], a brain tumor where HSP-27 has been indicated as a differentiation and prognostic marker[29]. Interestingly, quercetin shows antimalarial properties[30], and in human monocytes it has been reported to inhibit some HZ effects, such as enhancement of MMP-9 activity and TNFalpha/IL-1beta production[12]; on the other hand, doxorubicin is an antibiotic commonly used as antimalarial drug[1].

In conclusion, present work on THP-1 cells represents a useful premise for future HSP-27 silencing studies on a stable monocytic line, which are certainly required not only to investigate the role of HSP-27 in the detrimental survival of HZ-fed monocytes during complicated malaria, but also to explore if HSP-27 might be a potential target for new antimalarial therapies.

## Conflict of interest statement

The authors have no conflicting financial interests.

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