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Original Article

Comparative analysis of the interaction of HSPs in dendritic cells, macrophages, RGM-1 cells infected by *Helicobacter pylori*

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Abstract: *Helicobacter pylori* may cause chronic gastritis, even gastric cancer, however, antigen-presenting cells (APCs) are most important immune cells involved in the induction and expression of the underlying inflammatory responses to resist *H. pylori*. To study the interaction of HSPs in dendritic cells (DCs), macrophages and RGM-1 cells infected with *H. pylori*, HSP-27, HSP-60, HSP-70 and HSP-90 proteins were analyzed in the mucosa tissue or serum of gastritis patients caused by *H. pylori*, and in cell supernatant of DCs, macrophages, RGM-1 infected by *H. pylori*, or in above host cells. We found that HSP-27, HSP-60, HSP-70 and HSP-90 decreased in gastric epithelial cells, but increased significantly in DCs, macrophages. Meanwhile, inflammation associated proteins iNOS-2 and COX-2 were participated in the expression of HSPs in the process of host cells defensing against *H. pylori* infection. These findings contribute to understand the functions of HSP-27, HSP-60, HSP-70 and HSP-90 in *H. pylori* infection APCs and gastric epithelial cells indicating that HSPs would be diagnostic markers for *H. pylori* infection.

Keywords: HSPs, *Helicobacter pylori*, dendritic cells, macrophages, RGM-1

Introduction

Helicobacter pylori infection is the major cause of chronic gastritis, peptic ulcers and gastric cancer [1]. At present approximately half of the world's population is infected with this bacterium, however, only a minority of infection individuals develop gastric cancer [2, 3]. Gastric mucosa is protected by a complex defense system, which includes the production of surface mucus and bicarbonate, the regulation of gastric mucosal blood flow, the acceleration of epithelial regeneration, and the preservation of epithelial homeostasis to defense *H. pylori* infection [4-6].

HSPs are groups of stress-response proteins which are either constitutively expressed or induced through the transcriptional action of heat shock factor (HSF), which have been proved to play a cytoprotective role in gastrointestinal tract [7, 8]. HSPs are classified into four major families according to their biological activities and apparent molecular weights; HSP90, HSP70, HSP60 expressed constitutively, and small HSPs including HSP27 and HSP10 induced by various conditions, including heat, oxidative stress, or bacterium infection [9, 10]. A number of studies have consistently demonstrated that *H. pylori* infection delays gastric mucosal healing by disrupting the balance in cell apoptosis and proliferation, decreasing the migration of epithelial cells, decreasing blood flow or angiogenesis within the gastric mucosa, and HSPs could reverse these limitation and inferiorities in mucosal healing.

Dendritic cells (DCs), and macrophages are both myeloid antigen-presenting cells (APCs), and are present in the *H. pylori*-infected mucosa and are likely involved in both the induction and maintenance of *H. pylori* specific immune responses and inflammatory response. Macrophages or DCs were originated from a common myeloid progenitor, monocytes and could be differentiated into tissue when crossing the endothelial barrier and have a high capacity to

kill *H. pylori* [11, 12]. Dendritic cells, and macrophages are specialized in antigen capture, processing, and presentation to naïve T cells after migration to the draining lymph nodes [13], and are widely distributed in human tissues, including the gastric mucosa, and are capable of penetrating the gut epithelial monolayers to sample, e.g., luminal bacteria [14]. Exosomes derived from are dendritic cells, macrophages and tumor cells are lipid bilayer vesicles of 30 to 100 nm in size, which contain microbial components [15, 16] and could altered the expression of cellular inflammatory signaling proteins such as CD63, CD81 and HSP-90 [17].

Here, we intend to investigate the functions of HSPs in dendritic cells, macrophages, RGM-1 infected with *H. pylori* and the protective mechanisms of HSPs in *H. pylori* infection.

Methods

RGM-1, dendritic cells, macrophages, and bacterial growth conditions and preparations

RGM-1 cells purchased from the Cell Resource Center of Shanghai Academy of Sciences, Chinese Academy of Sciences were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), and amphotericin (100 U/ml), which were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Immature monocyte-derived DCs were generated by culturing 1×10^6 monocytes/ml in complete RPMI 1640 medium containing 2 mM L-glutamine (Gibco) and 10 mM HEPES (Gibco) supplemented with 10% FCS, and penicillin (100 U/ml)-streptomycin (100 g/ml) (Sigma) as well as 2,00 U/ml of granulocyte macrophage colony-stimulating factor (R&D Systems, MN) and 100 U/ml recombinant IL-4 (R&D Systems, MN). A total of 3×10^6 cells per well were cultivated in six-well culture plates. Fresh medium containing IL-4 and GM-CSF was added every second day to cell cultures. After six days, cells were harvested and washed once by fresh complete RPMI 1640 medium.

The human acute monocytic leukemia cell line THP-1 purchased from the Cell Resource Center of Shanghai Academy of Sciences, Chinese Academy of Sciences, were cultured in flasks at 37°C under 5% CO_2, in RPMI 1640-GlutaMAX™ (Gibco) containing 10% (v/v) fetal bovine serum (Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 amphotericin B (5 U/ml). Differentiation of THP1 cells into macrophage-like cells was induced by stimulation with 0.1 mM phorbol 12-myristate 13-acetate, PMA (Sigma) for 24 h.

The wild-type *H. pylori* strain 26695 obtained from the Cell Resource Center of Shanghai Academy of Sciences, Chinese Academy of Sciences was cultivated for 48 h at 37°C under microaerobic conditions (5% O_2) on selective agar consisting of 21.5 g of Wilkins Chalgren agar, 50 ml of human blood, vancomycin (100 U/ml), cefsulodin (100 U/ml), trimethoprim (100 U/ml) and amphotericin B (25 U/ml).

H. pylori co-cultured with RGM-1, dendritic cells, macrophages

RGM-1, dendritic cells, macrophages were washed twice with PBS and plated onto 24-well plastic plates at a density of 5×10^5 cells per well in 1 ml of RPMI-1640 growth medium, respectively. *H. pylori* were recovered from the agar plates using a swab and resuspended in RPMI-1640 growth medium at an optical density of 0.6 at 600 nm, which corresponds to 5×10^6 CFU/ml. The bacteria were added to RGM-1, dendritic cells, macrophages at the indicated multiplicity of infection (MOI) 10:1 and the co-cultures were further incubated at 37°C in a 5% CO_2 atmosphere for 24 h.

Acquisition of gastric tissues

Gastric tissues from patients such as gastric tissues of normal individual, gastric tissues of gastritis caused by *H. pylori* and gastric cancer tissues obtained from Kunshan First People's Hospital, Affiliated to Jiangsu University, The Second Xiangya Hospital of Central South University, and Chongqing Cancer Institute, which were approved by the ethical committee of Jiangsu University and Central South University.

Immunohistochemistry

Immunostainings for HSP-27, HSP-60, HSP-70 and HSP-90 in gastric tissues of normal individuals, gastritis caused by *H. pylori*, gastritis

and gastric cancer tissues were performed on paraffin-embedded tissue sections using commercially available goat anti-human HSP-27, HSP-60, HSP-70 and HSP-90 (BD, Franklin Lakes, NJ, USA) primary antibodies.

Briefly, each 3-μm tissue sections were deparaffinized and rehydrated. After rehydration through a graded ethanol series, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min for antigen retrieval, then cooled to 30°C and washed with phosphate-buffered saline (PBS, pH 7.3). After endogenous peroxidase had been quenched with aqueous 3% H_2O_2 for 10 minutes and washed with PBS, the sections were incubated at 4°C overnight with a HSP-27, HSP-60, HSP-70 and HSP-90 monoclonal mouse antibodies (R&D Systems, Minneapolis, MN, USA, 1:80 dilution) and then washed with PBS. Next, the sections were incubated with secondary antibody (R&D Systems, Minneapolis, MN, USA) for 30 min at room temperature. Two pathologists independently assessed the immunostained slides.

Exosomes isolation

Cell culture supernatants of RGM-1, dendritic cells, macrophages were centrifuged at 3,000 *g* for 20 minutes to remove cells and cell debris, then supernatants were transferred to sterile tubes. The ExoQuick Exosome Precipitation Solution (System Biosciences (SBI), Mountain View, CA, USA) was added to cell culture supernatants, the tubes mixed by inverting, then refrigerated for 30 minutes. ExoQuick/biofluid mixture was centrifuged at 1,500 *g* for 30 minutes, then the supernatant aspirated. Spin down residual ExoQuick solution was added and centrifuged at 1,500 *g* for 5 minutes and all traces of fluid removed by aspiration. Finally, exosomes pellets were resuspended in 1:10 of the original volume using nuclease-free water. The exosomes pellets were mixed with 20 μl of 9% sucrose containing protease inhibitors and stored at -80°C.

Western blotting

For western blotting, equal concentration of proteins from RGM-1, dendritic cells, macrophages infected by *H. pylori* or of exosomes from RGM-1, dendritic cells, macrophages infected by *H. pylori* , as quantitated by the Micro BCA Protein Assay, were loaded on 10% SDS-PAGE gels, electrophoresed, and transferred onto polyvinylidene difluoride membrane (Milipore, Bedford, MA). The membranes were probed for HSP-27 (1:400 dilution), HSP-60 (1:600 dilution), HSP-70 (1:500 dilution) and HSP-90 (1:800 dilution) (R&D Systems, Minneapolis, MN, USA). Immune-detected protein bands were quantified with Image J and statistically analyzed by ANOVA software.

Reverse transcription PCR (RT-PCR)

The primers designed according to the cDNA sequences of *iNOS-2* and *COX-2* and *GAPDH* obtained from the NCBI database. All primers were synthetized by Invitrogen (New York, US).

RT-PCR assays were performed using the Eppendorf PCR system (Eppendorf, Germany). Total RNAs were isolated from RGM-1, dendritic cells, macrophages followed *H. pylori* infection and the effect of noncytotoxic heat shock using TRIzol reagent and quantified spectrophotometrically. The cDNA was synthesized using M-MLV reverse transcriptase (Superscript-Invitrogen, Carlsbad, CA, USA). A reaction mixture containing 2 μL RNA (500 ng/μL), 12 μL 5×M-MLV RT Buffer, 6 μL 2.5 mM dNTP mixs, 1 μL RNase inhibitor (30 U/μL), 4 μL M-MLV Reverse Transcriptase (5 U/μL), 3 μL Oligo $dT_{(18)}$ primer (500 ng/μL) and 12 μL DEPC water was incubated at 37°C for 1 h. For RT-PCR, 1.0 μL cDNA, 0.5 μL forward primer and 0.5 μL reverse primer, 2 μL 2.5 mM dNTP mixs, 0.5 μL DNA polymerase (10 U/μL), 4 μL 5× Buffer and 11.5 μL ddH_2O were added into the system and amplified for 28 cycles under the following cycling conditions, 94°C denaturation 2 min, 28×(94°C for 20 s; 54°C, 25 s; 72°C for 20 s) extension at 72°C for 5 min.

Detection of cytokines

HSP-27, HSP-60, HSP-70 and HSP-90 in the culture supernatants of RGM-1, dendritic cells, macrophages infected by *H. pylori* and in the serum of normal individual, gastritis patients caused by *H. pylori*, gastritis patients and gastric cancer patients were monitored for cytokine levels by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Boster Biotechnology Company, WuHan, China). The results were read by using a SpectraMax 190 plate reader (Molecular Devices, CA) and were calculated by standard curves.

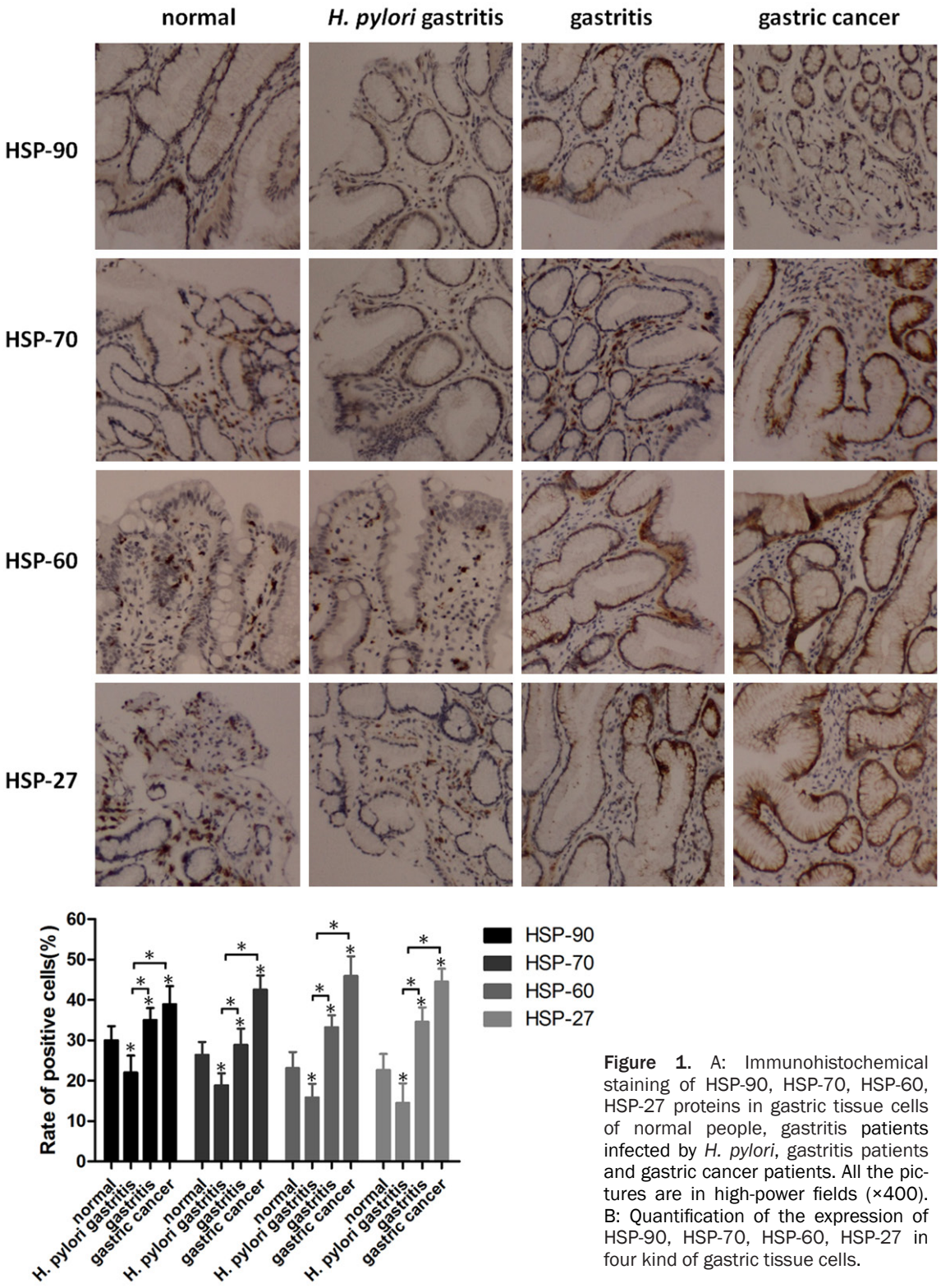

Figure 1. A: Immunohistochemical staining of HSP-90, HSP-70, HSP-60, HSP-27 proteins in gastric tissue cells of normal people, gastritis patients infected by *H. pylori*, gastritis patients and gastric cancer patients. All the pictures are in high-power fields (×400). B: Quantification of the expression of HSP-90, HSP-70, HSP-60, HSP-27 in four kind of gastric tissue cells.

Statistical analysis

Triplicates were performed in each experiment for each condition or group. Data were analyzed by SPSS 16.0 and are presented as means ± SEM of at least 3 independent experiments. Differences within each group were subjected to t-test or q-test. Statistically

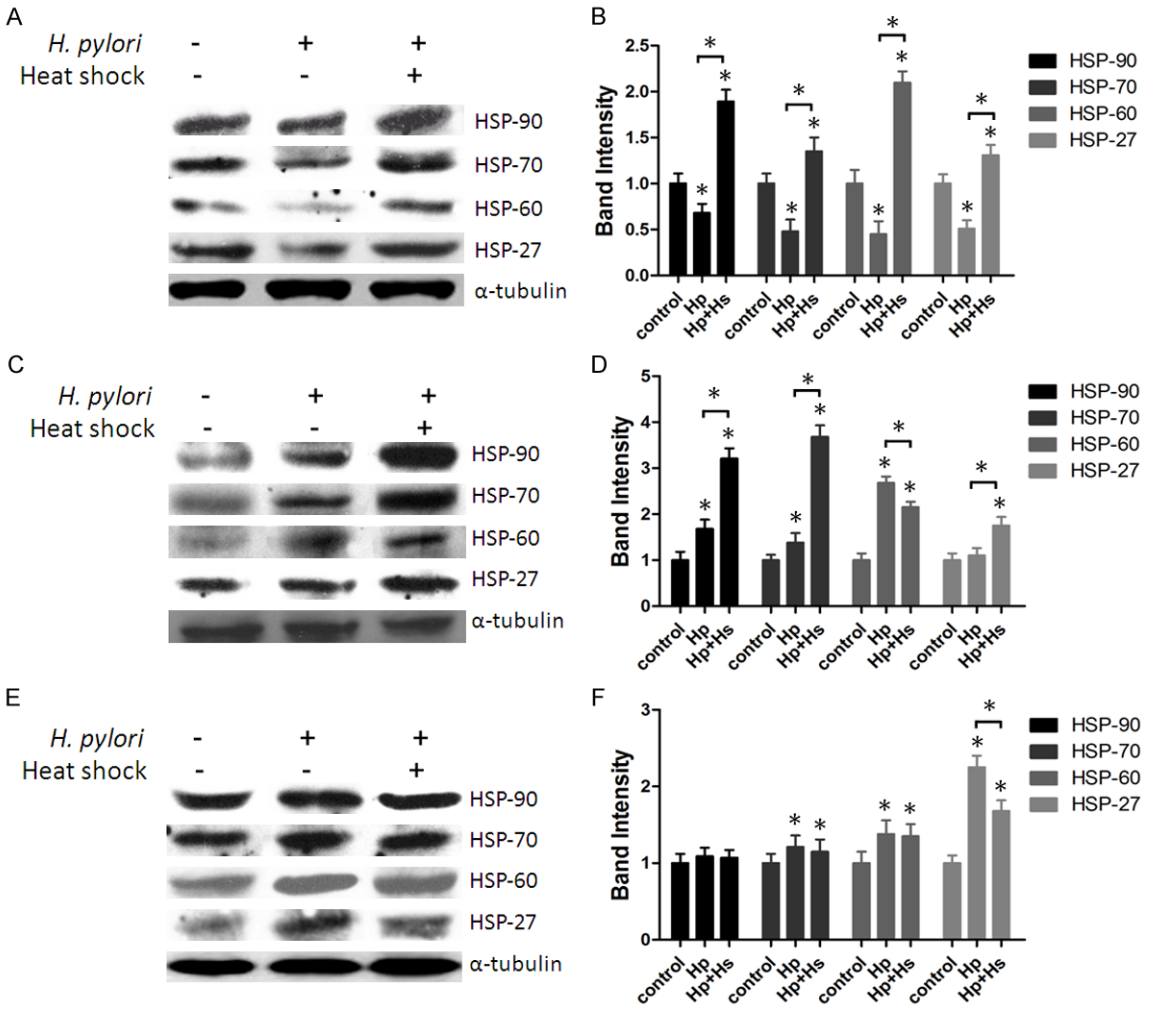

Figure 2. HSP-90, HSP-70, HSP-60, HSP-27 proteins in RGM-1 cells (A), macrophages (C), Dendritic cells (E) treated with *H. pylori* infection or heat shock were analyzed by western blotting. (A, C, E) Results of western blotting, α-tubulin as a standard calibration; (B, D, F) Quantified assay of HSP-90, HSP-70, HSP-60, HSP-27 analyzed by SPSS 16.0, with asterisks indicating the pairs of values compared for which significant differences were observed was presented as mean ± SEM. (n = 3 compared with the control group, **P*≤0.05 *vs* normal or control).

significant differences (**P*≤0.05) between the groups being compared are indicated by asterisks.

Results

The expression of HSPs proteins in gastric tissues

HSP-27, HSP-60, HSP-70 and HSP-90 proteins were all increased significantly in gastric tissue cells of gastritis patients and gastric cancer patients, on the contrary, they were decreased in gastric tissue cells of gastritis patients infected by *H. pylori* (**Figure 1**).

Analysis of HSPs proteins in RGM-1, dendritic cells, macrophages and exosomes

RGM-1, dendritic cells, macrophages were infected with *H. pylori*, then HSP-27, HSP-60, HSP-70 and HSP-90 in above cells (**Figure 2**) and in exosomes (**Figure 3**) from above cells were determined by western blotting. HSP-90 were up-regulated in macrophages infected with *H. pylori* and their exosomes, HSP-70 and HSP-60 were up-expressed in macrophages or dendritic cells infected with *H. pylori* and their exosomes. But, HSP-27 were increased in macrophages or dendritic cells infected with *H. pylori* and exosomes secreted from RGM-1,

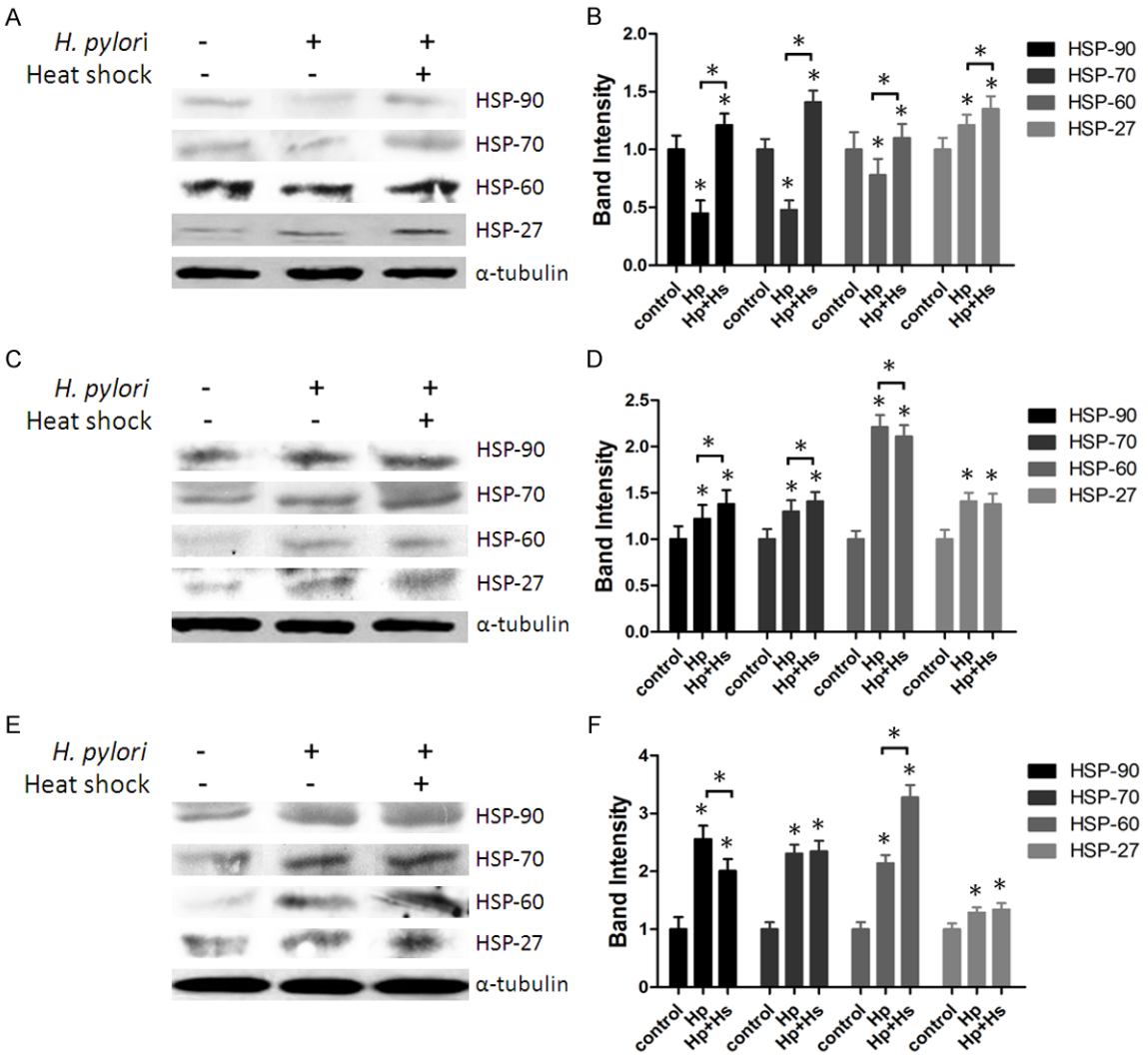

Figure 3. HSP-90, HSP-70, HSP-60, HSP-27 proteins in exosomes obtained from cell supernatant of RGM-1 cells (A), macrophages (C), Dendritic cells (E) stimulated with *H. pylori* or heat shock were analyzed by western blotting. (A, C, E) Results of western blotting, α-tubulin as a standard calibration; (B, D, F) Quantified assay of HSP-90, HSP-70, HSP-60, HSP-27 analyzed by SPSS 16.0, with asterisks indicating the pairs of values compared for which significant differences were observed was presented as mean ± SEM. (n = 3 compared with the control group, *$P \leq 0.05$ *vs* normal or control).

dendritic cells, macrophages infected with *H. pylori* obviously.

Oxygen/nitrogen metabolism

One of the antimicrobial defense mechanisms of host cell employed to eliminate invading pathogens is the use of reactive oxygen species and nitric oxide. Consequently, in the current study, we investigated whether *H. pylori* infection was also involved in enhanced oxygen/nitrogen metabolism through the measurement of two important enzymes (iNOS-2 and COX-2) in the metabolism process. As shown in **Figure 4**, *H. pylori* infection increased the expression of *iNOS-2* and *COX-2* at mRNA levels in RGM-1, dendritic cells, macrophages. These results indicated that *H. pylori* infection was involved in macrophage oxygen/nitrogen metabolism.

Proinflammatory cytokines analysis

Cytokines HSP-27, HSP-60, HSP-70 and HSP-90 in the cell supernatants of RGM-1, dendritic cells, macrophages infected by *H. pylori* (**Figure**

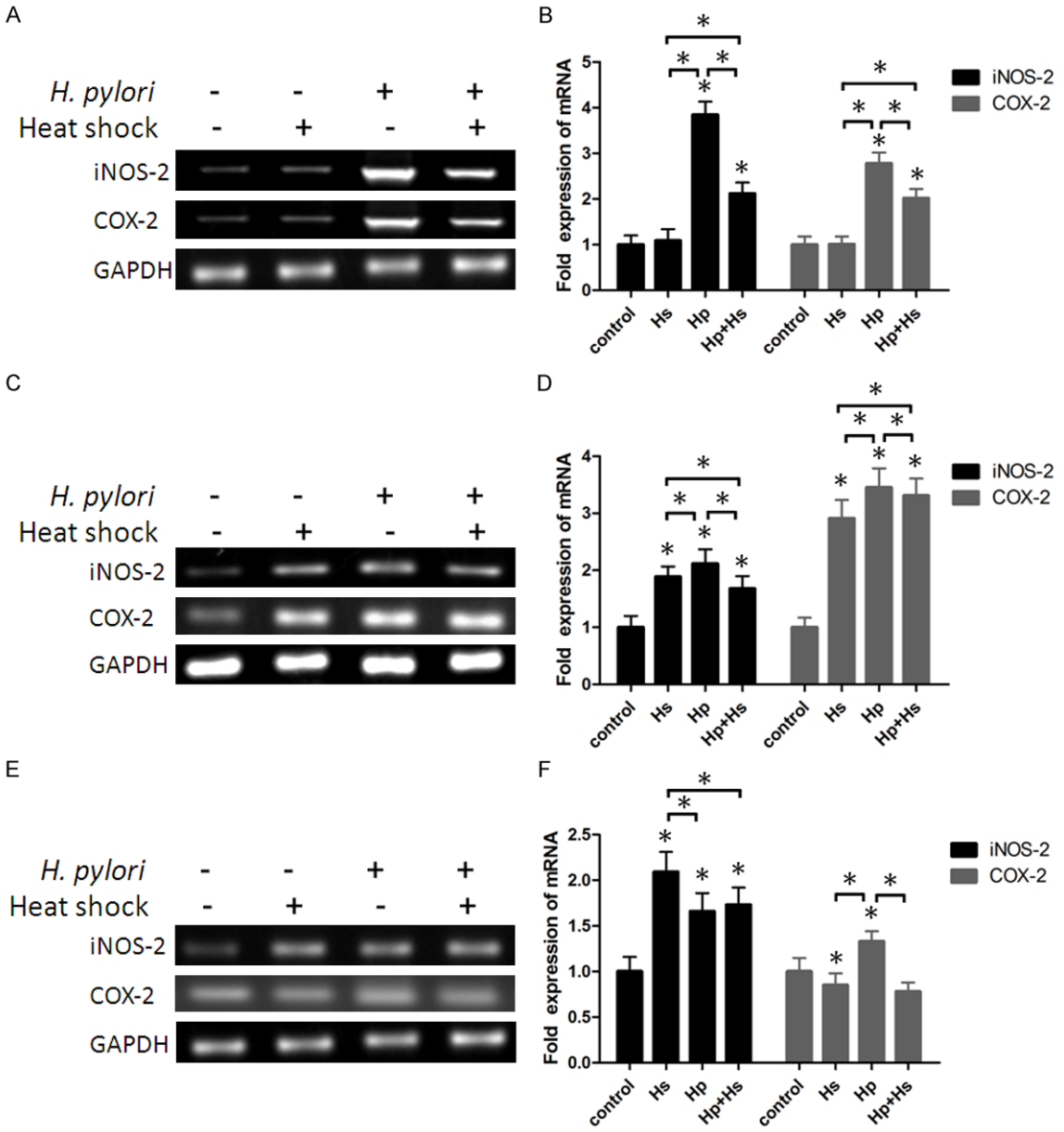

Figure 4. The expression of *iNOS-2* and *COX-2* genes following *H. pylori* infection and the effect of noncytotoxic heat shock. Total mRNAs of *iNOS-2* and *COX-2* isolated from *H. pylori*-infected or heat shocked RGM-1 cells (A), macrophages (C), Dendritic cells (E) were analyzed by RT-PCR. (B, D, F) Semi-quantification of *iNOS-2* and *COX-2* expression were conducted and data shown are mean ± SD of three independent experiments. One-way ANOVA plus SNK posttest were used for statistical analysis (*$P \leq 0.05$).

5) and in the serum of normal group, gastritis patients caused by *H. pylori*, gastritis patients, gastric cancer patients, were detected by ELISA kits (**Figure 6**). Notably, HSP-27, HSP-60, HSP-70 and HSP-90 were all increased significantly in the cell supernatants of dendritic cells, macrophages infected by *H. pylori*, but decreased in RGM-1 cells. Meanwhile, HSP-27, HSP-60, HSP-90 enhanced in the serum of *H. pylori* gastritis patients. Therefore, above results indicated that HSP-27, HSP-60, HSP-70 and HSP-90 in serum especially HSP-27, may be potential diagnostic markers for *H. pylori* infection patients.

Discussion

Heat shock proteins (HSPs) are ubiquitous, highly conserved proteins across all species, which are strongly induced by heat shock and

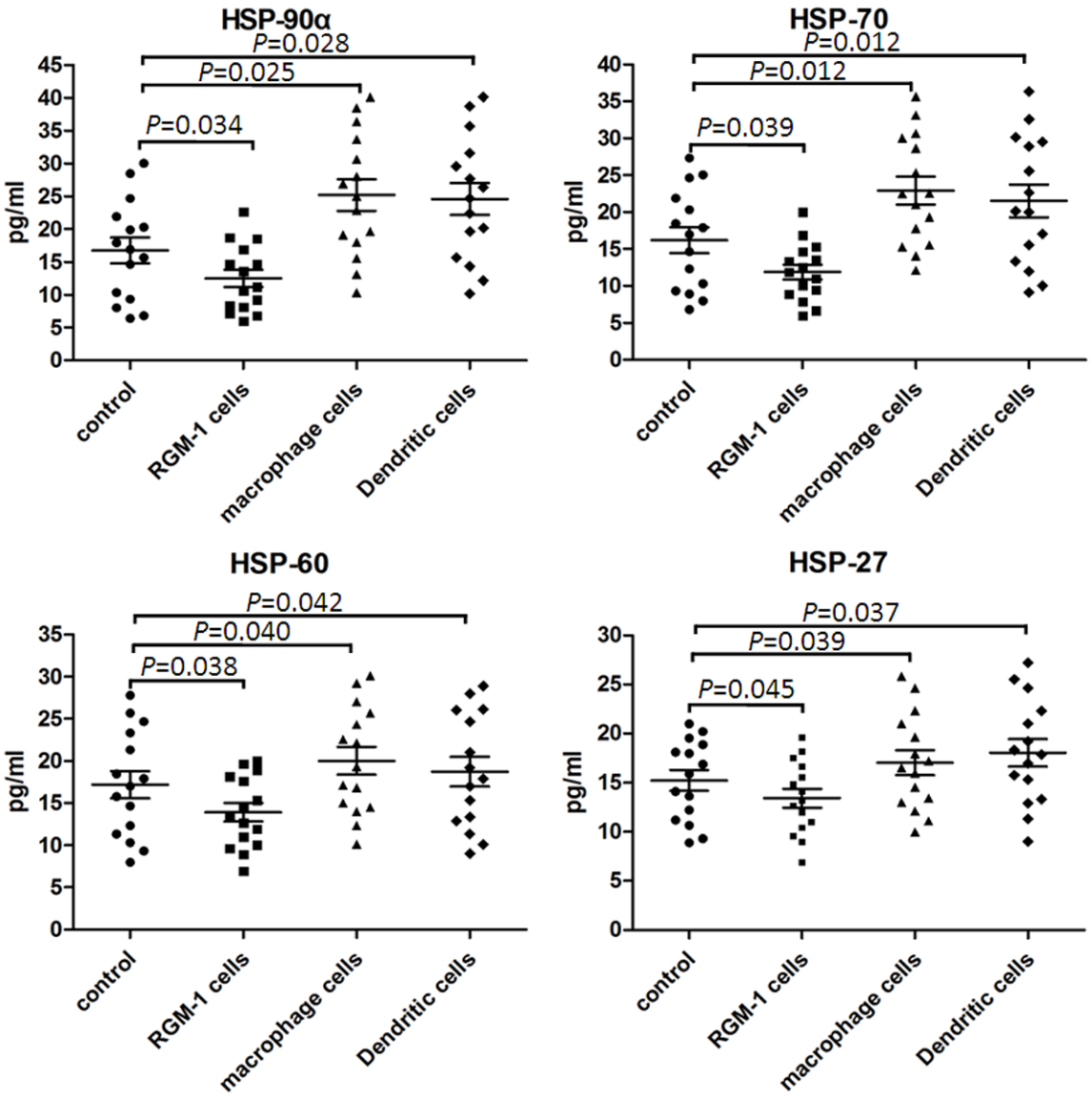

Figure 5. HSP-90α, HSP-70, HSP-60, HSP-27 in cell culture supernatants of RGM-1 cells, macrophages, Dendritic cells infected with *H. pylori* were determined by ELISA method. Each symbol per condition represents the data obtained from one tests. Horizontal lines show the median values of 15 experiments. $^{**}P \leq 0.05$ compared with medium alone (Friedman test and Dunn's multiple comparison test).

diverse environmental and physiopathological stresses [18, 19]. Apart from response to heat shock and chemical or physical stress stimuli, HSPs have been reported to be over-expressed in a wide range of human tumors including breast, endometrial, ovarian, colon, lung, prostate and gastrointestinal [20]. The expression of several HSPs has also been shown to correlate with tumor cell proliferation and differentiation, as well as apoptosis-related molecules in various types of cancer. Gastric cancer constitutes one of the most common malignant tumors in Asian countries, which remains a major health problem, representing the second cause of cancer-related deaths worldwide [21, 22]. However, *Helicobacter pylori* infection have been identified as a main environmental risk factor for gastric cancer [23]. Recent reports indicated that HSPs may have a close relationship with gastritis caused by *H. pylori*, but, there is little information about them. Present studies suggested that the expression

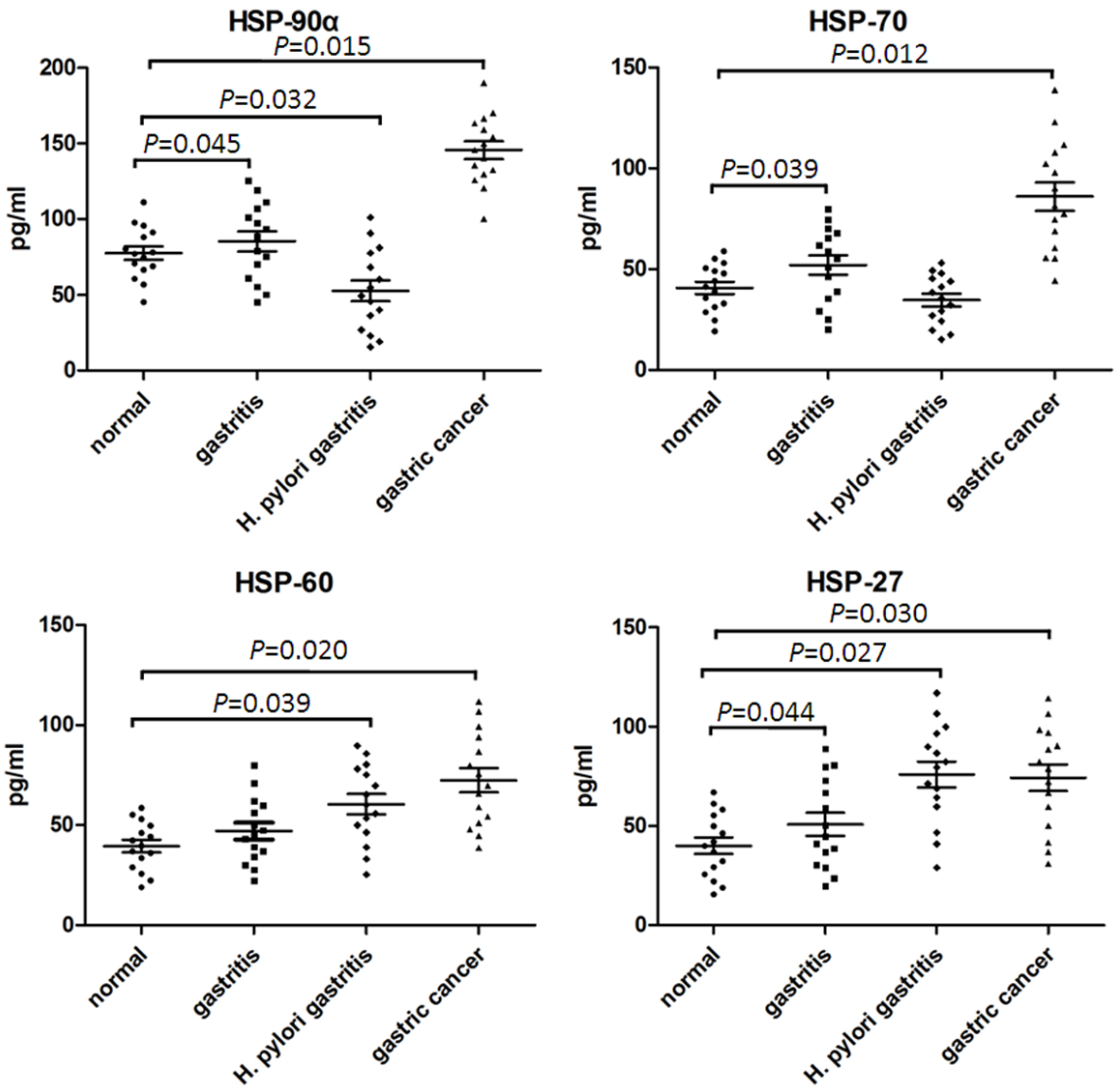

Figure 6. Analysis of HSP-90α, HSP-70, HSP-60 and HSP-27 in serum of normal individual, gastritis patients, *H. pylori* gastritis patients and gastric cancer patients was detected by ELISA method. Each symbol per condition represents the data obtained from one patients. Horizontal lines show the median values of 15 experiments. $^{**}P \leq 0.05$ compared with medium alone (Friedman test and Dunn's multiple comparison test).

of HSP-27, HSP-60, HSP-70 and HSP-90 proteins was associated with *H. pylori* infection in gastritis. All examined cases of gastritis and gastric cancer were tested positive for HSP-27, HSP-60, HSP-70 and HSP-90, and these HSPs proteins enhanced significantly compared with normal individual, interestingly, they were down regulated in *H. pylori* infection gastritis.

Gastric mucosa tissues including gastric epithelial cells, macrophages, dendritic cells are the first line of defense against *H. pylori* infection [24], within which HSPs are crucial for the maintenance of epithelial homeostasis during normal cell growth and for survival during and after various cellular stresses, supported with their molecular chaperone and cytoprotective actions like either protecting mitochondria or interfering with the stress-induced apoptotic programme [25]. In order to document the contribution of HSPs after *H. pylori* infection, first we performed western blot analysis in RGM-1 cells, macrophages, dendritic cells and exosomes secreted from above cells in order to check any shifts in HSP-27, HSP-60, HSP-70 and HSP-90 after *H. pylori* infection [26]. As

results, *H. pylori* infection significantly attenuated the expression of HSP-27, HSP-60, HSP-70 in RGM-1 cells and its exosomes, enhanced HSP-27, HSP-60, HSP-70 and HSP-90 in macrophages, dendritic cells and their exosomes.

Simultaneously, our RT-PCR results show that *iNOS-2* and *COX-2* genes were raised in RGM-1 cells, macrophages, dendritic cells infected by *H. pylori* suggestting that *iNOS-2* and *COX-2* were induced in RGM-1 cells by suppressing the expression of HSP-27, HSP-60, HSP-70 and HSP-90, which are major inflammatory mediators provoking *H. pylori* induced gastric tissue damage. Nevertheless, this mechanisms against *H. pylori* were opposite in macrophages, dendritic cells.

ELISA experiments about HSPs show that HSP-90α, HSP-70, HSP-60, HSP-27 increased significantly in cell culture supernatants of macrophages, Dendritic cells, but decreased in gastric epithelial cells RGM-1, however HSP-60 and HSP-27 increased observably in the serum of *H. pylori* gastritis patients. Several investigators including ours have published papers that HSPs were quite contributive in either acceleration of ulcer healing or prevention from recurrence, for which geranylgeranylacetone possessing evidences that HSPs may be a diagnostic marker of great value. Furthermore, prospective studies are necessary to elucidate the significance of HSPs in detecting early *H. pylori* infection.

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Disclosure of conflict of interest

None.

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