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Major Article

Strong renal expression of heat shock protein 70, high mobility group box 1, inducible nitric oxide synthase, and nitrotyrosine in mice model of severe malaria

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Abstract

Introduction: Renal damage is a consequence of severe malaria, and is generally caused by sequestration of *Plasmodium falciparum*-infected erythrocytes in the renal microcirculation, which leads to obstruction, hypoxia, and ischemia. This triggers high mobility group box 1 (HMGB1) to send a danger signal through toll-like receptors 2 and 4. This signal up-regulates inducible nitric oxide (iNOS) and nitrotyrosine to re-perfuse the tissue, and also increases heat shock protein 70 (HSP70) expression. As no study has examined the involvement of intracellular secondary molecules in this setting, the present study compared the renal expressions of HSP70, HMGB1, iNOS, and nitrotyrosine between mice suffered from severe malaria and normal mice. **Methods**: C57BL/6 mice were divided into an infected group (intraperitoneal injection of 10^6 *P. berghei* ANKA) and a non-infected group. Renal damage was evaluated using hematoxylin eosin staining, and immunohistochemistry was used to evaluate the expressions of HSP70, HMGB1, iNOS, and nitrotyrosine. **Results**: Significant inter-group differences were observed in the renal expressions of HSP70, HMGB1, and iNOS (p=0.000, Mann-Whitney test), as well as nitrotyrosine (p=0.000, independent t test). The expressions of HSP70 and HMGB1 were strongly correlated (p=0.000, R=1.000). No correlations were observed between iNOS and HMGB, HMGB1 and nitrotyrosine, HSP70 and nitrotyrosine, or iNOS and nitrotyrosine. **Conclusions**: It appears that HMGB1, HSP70, iNOS, and nitrotyrosine play roles in the renal damage that is observed in mice with severe malaria. Only HSP70 expression is strongly correlated with the expression of HMGB1.

Keywords: HMGB1. HSP70. iNOS. Nitrotyrosine. Severe malaria.

INTRODUCTION

According to the World Health Organization, approximately 3.2 billion people were at-risk of malaria in 2015, with 214 million new cases in 106 countries. Among the new cases, approximately 88% were located in Africa, followed by Southeast Asia (10%) and the East Mediterranean (2%). There were approximately 438,000 deaths related to malaria in 2015, with 90% occurring in Africa, 7% occurring in Southeast Asia, and 2% occurring in East ekMediterranean[1]. As malaria is endemic in Indonesia, approximately 70% of the Indonesian population is at-risk and approximately 38,000 deaths are caused by malaria every year, with most occurring in east Indonesia (80%)[2].

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The most common cause of severe malaria is *Plasmodium falciparum* infection[3], and acute kidney injury (AKI) is a common manifestation of severe malaria[4]. Infected erythrocytes express *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) and *P. falciparum* histidine-rich protein (PfHRP), which play roles in knob formation. In addition, the infected erythrocytes easily adhere to endothelial cells using PfEMP1, which binds to endothelial adhesive factors, such as thrombospondin, intercellular adhesion molecule 1 (ICAM), vascular cell adhesion molecule (VCAM), and endothelial leukocyte adhesion molecule 1 (ELAM-1)[5]. In cases of severe malaria, the parasite also produces glycophosphatidylinositol (GPI), which is a toxin that induces a renal cytoadherence process that leads to pro-inflammatory cytokine production in an effort to eliminate the parasite. These processes lead to renal hypoxia and ischemia, and subsequently to AKI[6,7], although the precise pathway remains unclear. It is possible that the infected and easily adherent erythrocytes form clots of infected and non-infected erythrocyte (rossetting), which could cause

the circulatory obstruction, hypoxia, and ischemia. It is also possible that the damage is mediated by immune complexes that are deposited in the glomerulus. Alternatively, the damage could be related to a hemodynamic disturbance that is caused by dehydration and hypovolemia, as well as alteration of the renal microcirculation[8]. Prolonged hypoxia generates alternate oxidative phosphorylation product coupling in the mitochondria and depletion of adenosine triphosphate (ATP) stores as adenosine is metabolized into inosine and hypoxanthine. The depletion of ATP subsequently leads to cellular edema, increased osmotic pressure, and cellular decompartementilization[9,10], which induces high mobility group box 1 (HMGB1) to generate a danger signal through toll-like receptors 2 and 4[11]. This signal strongly promotes the production of inflammatory cytokines, which increase renal stress and stimulate resident macrophages that can destroy renal tissue[12].

During stressful and hypoxic conditions, the body activates inducible nitric oxide (iNOS) to catalyze the production of nitric oxide (NO) and peroxynitrite, which act as vasodilators and help re-perfuse the hypoxic tissue[13]. Peroxynitrite production can be measured using nitrotyrosine levels[14]. The body also increases the production of heat shock protein 70 (HSP70), which is a chaperone protein with many injury-related functions[15], such as repairing unfolded or mis-folded protein, anti-inflammatory effects, stimulating regulatory T-cells, and stabilizing the cytoskeleton by repairing its organelle and architecture. In addition, HSP70 prevents further renal damage and restores renal function[16]. The present study aimed to compare the expressions of HSP70, HMGB1, iNOS, and nitrotyrosine in the renal tissue of mice that were and were not infected with *P. berghei* ANKA. This information will be useful in examining these molecules' involvement in renal injury that is caused by severe malaria.

METHODS

Animals

The study's protocol was approved by the ethical committee of our Faculty of Medicine (Universitas Brawijaya, Malang; 410/EC/KEPK/07/2014, date: July 10, 2014), and the study was performed between April 2014 and September 2015 in Parasitology Laboratory and Biomedical Laboratory Faculty of Medicine, Universitas Brawijaya, Malang. Eighteen female C57BL/6 mice (weight: 20-25g, age: 12-16 weeks) were purchased from the Eijkman Institute Jakarta. The mice were then divided into a non infected group (9 mice) and a group of 9 mice that were infected with 10^6 *P. berghei* ANKA in 0.2mL of blood via an intra-peritoneal injection.

Evaluating parasitemia

Parasitemia was evaluated daily until day 14 using thin blood smears. A previous study revealed that pro-inflammatory mediators are increased at day 7 and that pro-inflammatory hypercellularity peaks on days 14-15[17]. Infected erythrocytes were counted in a population of 1,000 erythrocytes using microscopy (magnification: ×1,000), and the counted number was converted into a percentage (%).

Hematoxylin and eosin staining

On day 14, the mice were anesthetized using chloroform and then sacrificed before the kidneys were collected and preserved in 10% formalin. Histopathology using hematoxylin and eosin staining were performed at the Pathology Anatomy Laboratory of Dr. Soetomo Hospital, Surabaya. The kidney specimens were dehydrated for 30 min at each step of an alcohol gradient: 30%, 50%, 70%, 85%, 95%, and twice at 100%. Clearing was performed twice using xylol for 1h. The specimens were subsequently immersed twice in liquid paraffin (1h at 42-46°C), blocked using solid paraffin in a paraffin cast, and incubated at 46-52°C for 1 day. Deparaffinization was performed for 5 min at each step of an alcohol gradient: 30%, 50%, 70%, 85%, 95%, and twice at 100%. The specimens were subsequently rinsed twice using H_2O for 5 min, stained using hematoxylin and eosin, and then mounted using 5% gelatin.

Immunohistochemistry

The immunohistochemistry was performed in our Biomedical Laboratory (Faculty of Medicine, Universitas Brawijaya). The slides were incubated at 60°C for 1h, and then sequentially treated using the following solutions: xylol (2× 10 min), absolute ethanol (2× 10 min), 90% ethanol (1× 5 min), 80% ethanol (1× 5 min), 70% ethanol (1× 5 min), and sterile distilled water (3× 5 min). Antigen retrieval was performed by heating the slides in a water bath at 95ºC for 20 min, cooling the slides to room temperature for 20 min, and then soaking the slides in a citric buffer chamber (pH 6.0). The slides were subsequently washed using phosphate-buffered saline [(PBS); 3× 2 min], treated using 3% H_2O_2 in methanol, incubated for 15 min, and then washed using PBS (3× 2 min). Blocking was performed using a background sniper solution (15 min at room temperature), and then the slides were washed using PBS (3× 2 min).

The antibodies that targeted NOS2 (N-20, sc-651), nitrotyrosine (39B6, sc-32757), and HMG-1 (J2E1, sc: 135809) were manufactured by Santa Cruz Biotechnology. The antibodies to HSP70 (2A4) were manufactured by Stress Marq Biosciences. The slides were incubated with the primary antibodies, PBS buffer, and 2% BSA overnight at 4°C. On the next day, the slides were rinsed using PBS (3× 2 min), incubated with secondary antibodies for 30 min at room temperature, and then rinsed using PBS (3× 2 min). The slides were subsequently incubated with horseradish peroxidase-conjugated streptavidin for 20 min at room temperature, and then rinsed using PBS (3× 2 min) and then distilled water. Next, the slides were incubated with diaminobenzidine (DAB) and DAB buffer (1:50) for 3-10 min at room temperature, and then washed using PBS (3× 2 min) and distilled water (3× 2 min). Finally, the slides were rinsed using a Mayer and tap water solution (1:10), incubated for 5-10 min at room temperature, rinsed using tap water, dried, and observed under a microscope (magnification: ×1,000).

Histopathological examination

Renal damage can be histopathologically observed in the medulla and cortex as glomerulus necrosis, inflammatory cell infiltration, mesangial cell proliferation, and glomerulus

sclerosis. The expressions of HSP70, HMGB1, iNOS, and nitrotyrosine were measured by counting dark brown cells in 20 fields of view at ×1,000 magnification (quantitative scoring: 3+ to 4+). The evaluations were performed using a light microscope (Nikon Eclipse Ci), a calibrated camera (Optilab Plus 12 Megapixel), and Raster 3 software.

Statistical analysis

Normality was evaluated using the Kolmogorov-Smirnov test, and sample homogeneity was evaluated using the Levene test. Protein expressions were compared between the infected and non-infected groups using the Mann-Whitney and independent t tests. Spearman and Pearson correlation testing was used to evaluate correlations between the protein expressions. All data were analyzed using International Business Machines Statistical Package for the Social Sciences (IBM SPSS) software (version 22.0; IBM Corp., Chicago, IL), and differences were considered statistically significant at a p-value of <0.05.

RESULTS

Figure 1 shows the degree of parasitemia over time and the parasites' morphology. The infected group exhibited histopathological evidence of glomerular damage, including glomerular necrosis, inflammatory cell infiltration, mesangial proliferation, deposition of hemozoin pigment, and glomerular sclerosis (data not shown).

Immunohistochemical findings

Expressions of HMGB1, HSP70, iNOS, and nitrotyrosine were predominantly observed in the cytoplasm of glomerular and tubular cells (**Figure 2, Figure 3, Figure 4** and **Figure 5**). When the infected and non-infected groups were compared, the Mann-Whitney test revealed significant differences in the expressions of HSP70 (5.344 vs. 0.039; p=0.000), HMGB1 (4.439 vs. 0.089; p=0.000), and iNOS (6.533 vs. 0.006; p=0.000). In addition, the independent t test revealed a significant difference in the expression of nitrotyrosine (5.872 vs. 0.717; p=0.000).

The Spearman correlation test results revealed a significant correlation between the expressions of HSP70 and HMGB1 (p=0.000, R=1.000). However, there was no correlation between iNOS and degree of parasitemia (p=0.501, R=-0.259), nitrotyrosine and degree of parasitemia (p=0.189, R=0.482), HSP70 and degree of parasitemia (p=0.212, R=-0.461), HMGB1 and degree of parasitemia (p=0.212, R=0.461), iNOS and HMGB1 (p=0.244, R=-0.433), iNOS and HSP70 (p=0.244, R=-0.433), HMGB1 and nitrotyrosine (p=0.406, R=0.317), HSP70 and nitrotyrosine (p=0.406, R=0.317), or iNOS and nitrotyrosine (p=0.154, R=0.517).

DISCUSSION

The infected group exhibited a significant increase in parasitemia during day 5-10, which may have been related to parasite proliferation, and a subsequent decline starting on day 12, which could reflect the effects of the inflammatory process and immune response. However, the pro-inflammatory hypercellularity failed to eliminate the parasite, and the parasitemia subsequently increased significantly until day 14. During this period, we detected histopathological evidence of glomerular necrosis, infiltration of polymorphonuclear and mononuclear cells, mesangial proliferation, hemozoin deposition, and tubular edema. These findings are similar to the histopathological profile of acute renal failure caused by malaria, which manifests as acute tubular necrosis, interstitial nephritis, and glomerulonephritis[18]. Furthermore, glomerulonephritis can be characterized by glomerular cell proliferation (e.g., mesangial, podocyte, and endothelial cells) as well as changes in the basal membranes, inflammatory cell infiltration, deposition of hemozoin, presence of infected erythrocytes in the glomerulus capillaries, necrosis, and deposition of immune complexes[19]. The glomerular cell proliferation may be related to the secondary immune response to cytokines that are released by the host in response to the infection[20].

The present study revealed a significant difference in the infected and non-infected groups' expressions of HSP70, which is consistent with the theory that HSP70 expression is increased in cases of malaria. Interestingly, hemolysis is involved in the pathogenesis of malaria, as Deuel et al. observed significant increases in the genetic regulation of HSP70 and HMOX1 expression in proximal tubular cells after heme exposure[21,22]. In this context, the release of hemoglobin (Hb) causes endothelial dysfunction, oxidative vascular toxicity, and renal failure through several mechanisms[23]. First, Hb can rapidly convert into a heterodimer that can exit the blood vessels and enter various organs, including the kidneys. Second, Hb interacts with various non-oxygen ligands, such as NO and peroxides, which can lead to oxidative tissue damage. Third, ferritin Hb (Fe^{3+}) is a product of the hemoglobin auto-oxidation reaction or the reaction of Hb with endogenous oxidants, which can release free heme and lead to lipid peroxidation and subsequently inflammation. Furthermore, high concentrations of heme can trigger unfolded protein receptors[24].

During normal physiological conditions, the amount of free hemoglobin is controlled by plasma protein and haptoglobin, although only glomerular filtration can remove free hemoglobin from the circulation during pathological conditions. This can lead to short-term exposure to high concentrations of free hemoglobin, which can subsequently lead to AKI[25]. The body uses HSP70 over-expression as a protective mechanism[15], which can help protect the kidneys from ischemic injury[16].

The present study also revealed a significant infection-related difference in the expression of HMGB1, which can be passively released by ischemic and necrotic cells, and actively released by macrophages. Furthermore, the *Plasmodium* genome encodes two types of HMGB (HMGB1 and HMGB2), and the release of these proteins from red blood cells is suspected to contribute to the pathogenesis of AKI. For example, Wu et al. reported that HMGB1 expression was increased in AKI, and reportedly causes kidney damage through activation of the innate immune system[26]. In this context, HMGB1 triggers cellular signaling by binding to TLR4, which activates NFkB through a MyD88-dependent pathway and initiates the inflammatory response

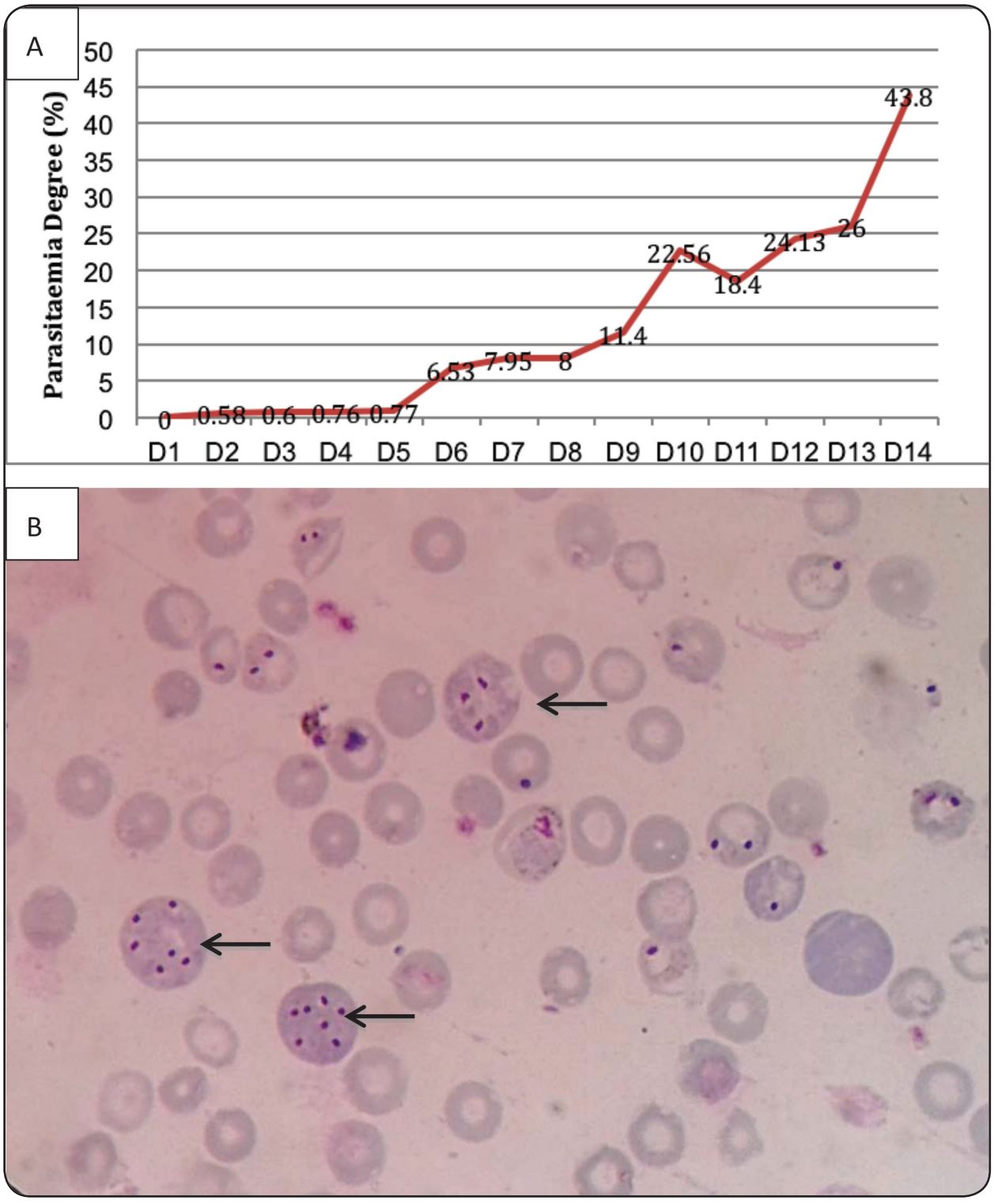

FIGURE 1 - A: Mean parasitemia between day 1 and day 14 after the inoculation. **B:** A thin blood smear from a mouse infected with *Plasmodium berghei* on day 14 reveals mature schizont (arrows).

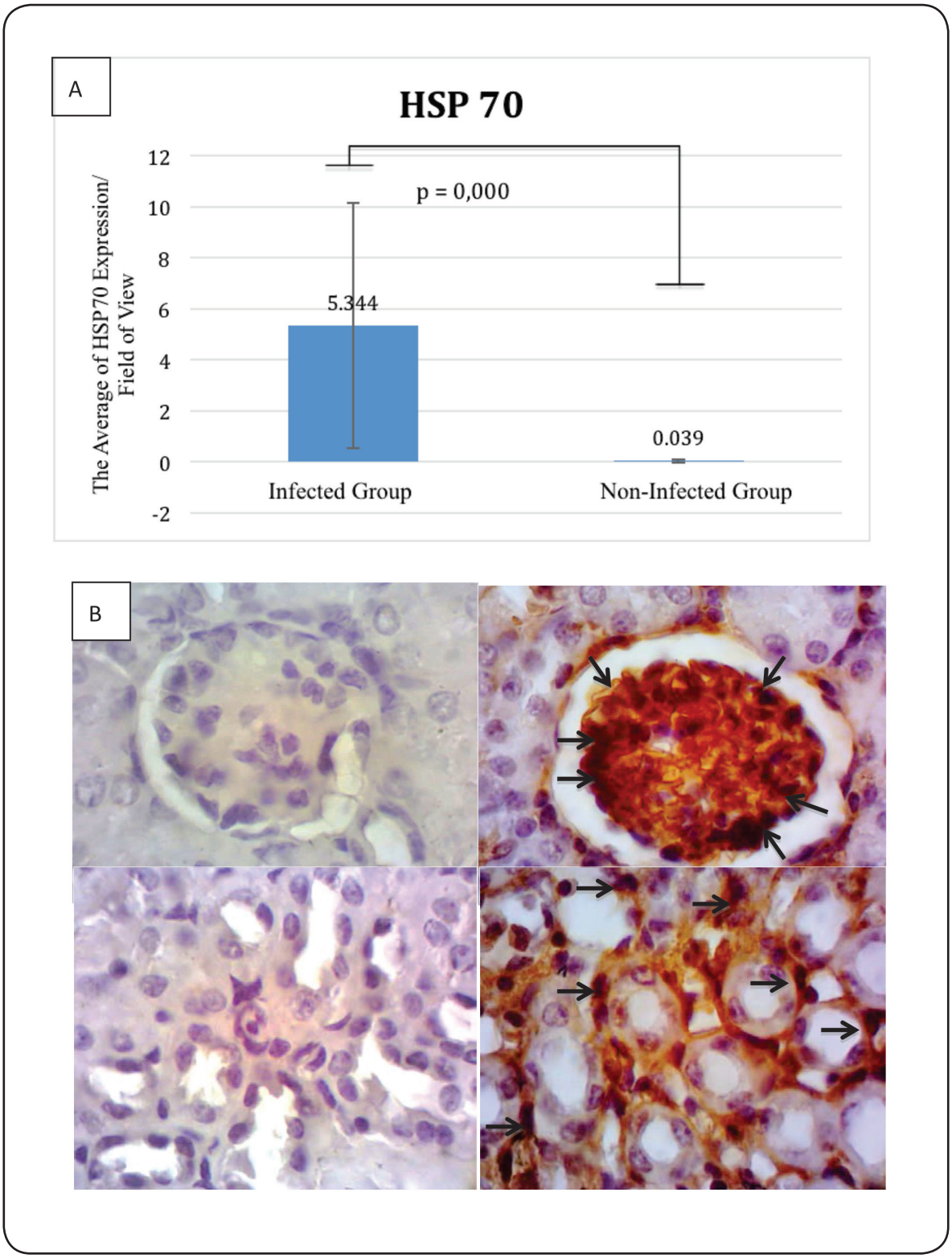

FIGURE 2 - A: HSP70 expression (positive cell) per field of view. **B:** The expression of HSP70 in the non-infected group (left, ×1,000) and infected group (right, ×1,000). HSP70 was expressed by glomerular cells and tubular cells (dark brown cells, black arrow) in the infected group. **HSP70:** heat shock protein 70.

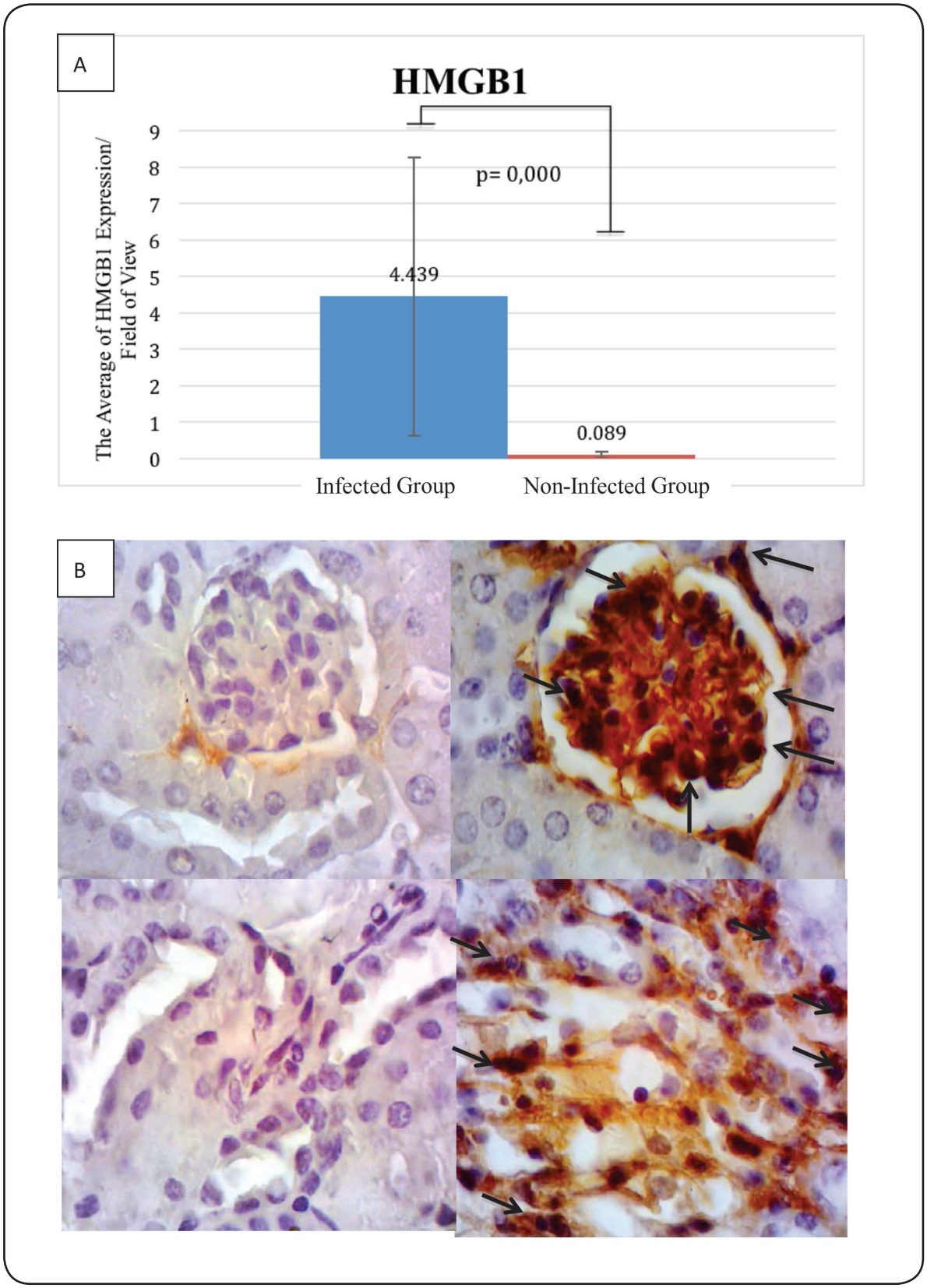

FIGURE 3 - A: HMGB1 expression (positive cells) per field of view. **B:** The expression of HMGB1 in the non-infected group (left, ×1,000) and infected group (right, ×1,000). HMGB1 was expressed by glomerular cells and tubular cells (dark brown cells, black arrows) in the infected group. **HMGB1:** high mobility group box 1.

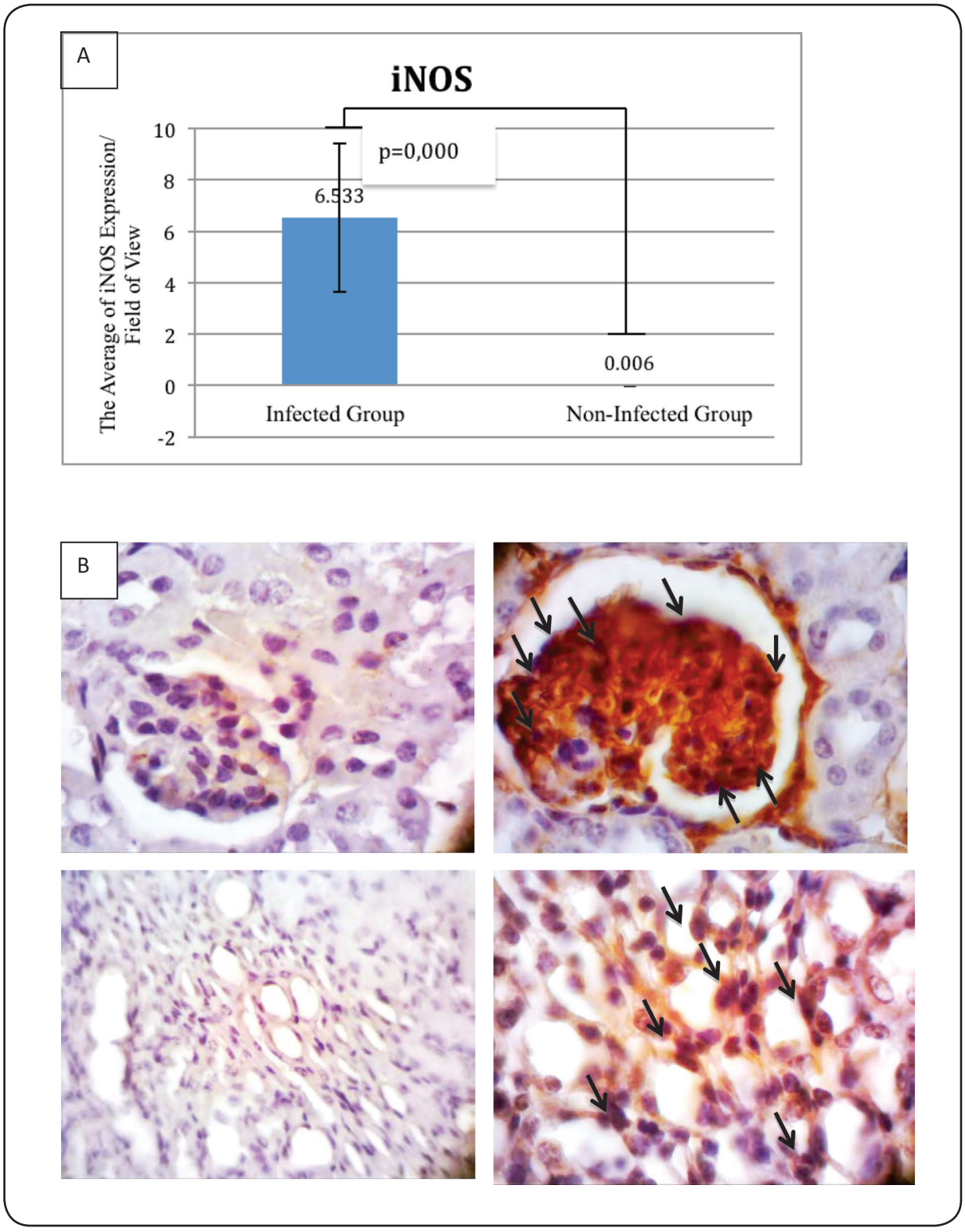

FIGURE 4 - A: iNOS expression (positive cells) per field of view. **B:** The expression of iNOS in the non-infected group (left, ×1,000) and infected group (right, ×1,000). iNOS was expressed by glomerular cells and tubular cells (dark brown cells, black arrows) in the infected group. **iNOS:** inducible nitric oxide.

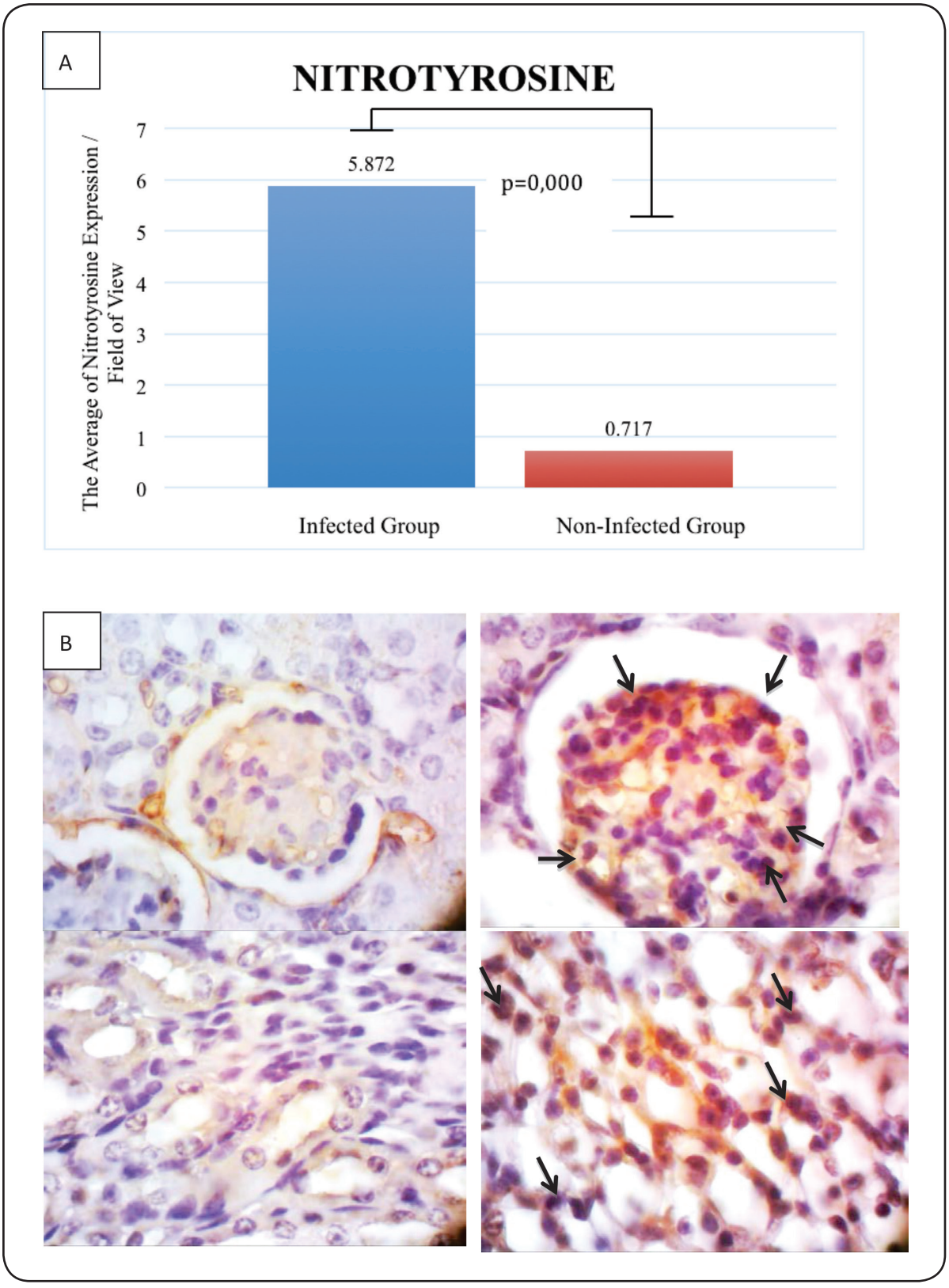

FIGURE 5 - A: Nitrotyrosine expression (positive cells) per field of view. **B:** The expression of nitrotyrosine in the non-infected group (left, ×1,000) and infected group (right, ×1,000). Nitrotyrosine was expressed by glomerular cells and tubular cells (dark brown cells, black arrows) in the infected group.

by releasing pro-inflammatory cytokines and chemokines[27]. Moreover, HMGB1 directly induces prothrombin expression on endothelial cells, which can contribute to the inflammatory response and exacerbate any renal impairment[12].

Spearman testing revealed a very strong correlation between the expressions of HSP70 and HMGB1 in the infected group, which suggests that these two variables were practically equal in the sample rank results. However, it is very common to have perfect correlation in a small sample size, as the Spearman test uses a rank system to evaluate correlation. During the inflammatory process, HMGB1 expression is associated with tissue damage (i.e., a damage-associated molecular protein) and is influenced by abiotic stressors, such as heat[28]. In addition, Mezayen et al. found that triggering expressed on myeloid cells-1 (TREM-1) is an HMGB-1 mediator that induces cytokine production, which suggests that HMGB-1 is released as an endogenous danger signal that induces the pro-inflammatory response of macrophages and leads to exacerbated kidney damage[29]. However, HSP70 expression increases in the presence of pathogenic stressors, such as heat, ischemia, and elevate levels of pro-inflammatory cytokines that are induced by the expression HMGB1. Thus, HSP70 can protect the kidneys from severe renal damage[16] by interfering with macrophages' pro-inflammatory response and the mis-folding of proteins[30]. In addition, expressions of HSP70 and HMGB1 are induced sequentially, although the mechanisms of their regulation and the relationship between these molecules remain unknown[28].

The infected and non-infected group had significantly different expressions of iNOS, and Elias et al. also observed similar results in BALB/C mice that were infected with *P. berghei*. This difference may be related to the cytoadherence of erythrocytes that are infected with *P. berghei*, which leads to interactions between the parasite antigens and endothelial receptors[17]. For example, infected erythrocytes can bind to ICAM-1, which is upregulated when endothelial cells are exposed to oxidative stress and parasites, and plays an important role in the microvascular dysfunction that can lead to increased renal expression of iNOS[31,32]. Thus, Elias et al. concluded that oxidative stress and pro-inflammatory cytokines play an important role in the pathogenesis of acute renal failure that is caused by *P. berghei* infection[17].

Although we detected a significant difference in the expression of nitrotyrosine, there is limited information regarding the expression of nitrotyrosine in models of *Plasmodium* infection. However, nitrotyrosine is a marker that is used to detect peroxynitrite (ONOO/ONOOH), which is mainly formed by the reaction of superoxide radicals with NO. Furthermore, reactive oxygen species (ROS) are produced during parasite vacuole digestion in infected erythrocytes, which can induce the rapid conversion of oxyhemoglobin into methemoglobin, as well as the production of H_2O_2[14,32].

We did not detect correlations between the expressions of iNOS and nitrotyrosine, or between their expressions and the degree of parasitemia ($p>0.05$). Elias et al. have suggested that parasitemia can be detected on day 3 and increases until day 14 or the last day of the study period[17]. However, no significant change was observed in iNOS expression during days 3-12, although a general positive trend was observed during the study period. Thus, it appears that the maximum expression of iNOS and other markers occurs at approximately 3 days after *Plasmodium* infection. Although no exponential increases were observed in the expression values, it is also possible that the expressions will continue to increase, unlike the degree of parasitemia[29]. Furthermore, the degree of parasitemia is a weak predictor of malaria severity and mortality, as it is not related to the levels of inflammation and organ damage. In contrast, infected erythrocyte sequestration is a strong predictor of malaria severity and vital organ damage (e.g., the brain in cases of cerebral malaria). Moreover, erythrocyte sequestration and malaria severity are associated with microvascular congestion by infected and uninfected erythrocytes[33]. Another study also found that there was no association between the degree of peripheral parasitemia and the incidence of acute renal failure[34].

Previous studies have indicated that NO and nitrotyrosine expression are related, although NO does not appear to be directly related to the expression of HSP70 or HMGB1. In this context, the direct interaction between ROS and NO produces nitrotyrosine and peroxynitrite, and the reaction of peroxynitrite with tyrosine can also generate nitrotyrosine. In addition, ROS can activate tyrosine residues to produce tyrosyl radicals that oxidize NO to produce nitrotyrosine. As nitrotyrosine can be formed by the reaction of tyrosine with other reactive molecules, the absence of a correlation between iNOS and nitrotyrosine is likely related to the multi-pathway production of peroxynitrite. However, the present study only considered two products of these pathways, and the dominant pathway remains unclear[35].

In conclusion, the expressions of HMGB1, HSP70, iNOS, and nitrotyrosine were elevated in mice model of AKI that is caused by severe malaria. However, only HSP70 expression was strongly correlated with the expression of HMGB1, with the expression of HMGB1 preceded the increased expression of HSP70. The design of the present study was not sufficient to determine the causality of this relationship, and the findings are also limited by the lack of a test for renal function and AKI severity. Moreover, there were no correlations between the degree of parasitemia and the expressions of HSP70, HMGB1, iNOS, or nitrotyrosine.

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Conflicts of interest

The authors declare that they have no conflicts of interest .

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