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Intestinal oxidative damage and mucin regulated gene response to *Plasmodium chabaudi* malaria infection.

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Abstract

Malaria, initiated inevitably by examining those apicomplexan parasites Plasmodium, is a huge purpose behind horribleness likewise mortal adversary all around the world particularly in developing countries.

The current study aimed to investigate the mice intestinal oxidative damage and mucin regulated gene response to *Plasmodium chabaudi* infection. The infection of female C57BL/6 mice with 10^6 *P. chabaudi* parasitized erythrocytes induced a maximum parasitemia (about 47 %) on day 8 p.i.. Histological examination of stained sections showed some defects in jejunal tissue of mice infected with *P. chabaudi* were injured and contained marked inflammatory cells. In addition, examination of Alcian blue stained sections demonstrated a significant increase in the number of goblet cells of the jejunal villi. Moreover, the infection induced a significant increase in the level of NO and MDA while the level of GSH was significantly decreased by the infection. Upon infection with *P. chabaudi*, there was a significant increase in the mRNA expression of MUC2 and MUC4. Based on our results, we can conclude that, the intestinal response to *P. chabaudi* infection could help in understanding the process of intestinal oxidative damage as well as the role of mucin related genes during infection. Further studies are required to know the mechanism and the pathway by which the parasite induce these intestinal alterations.

Keywords: Intestine, Mice, *Plasmodium chabaudi*, Oxidative stress, Goblet cells.

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Introduction

Malaria is a dangerous disease that is transmitted to people through the insect bite of infected mosquitoes with plasmodium [1]. World health organization reported that, about 3.4 billion people live in areas at risk of malaria transmission in 106 countries and territories. In 2013, it was reported that malaria caused 198 million clinical episodes, and 500,000 deaths [2].

The intestine is not directly affected by malaria, although abdominal pains and nausea are symptoms of malaria. This may be due to the infection induced fever. During the infection of human with Plasmodium, the capillaries of the intestinal villi could be blocked with parasitized erythrocytes [3] and are associated with gastrointestinal injury with defects in metabolism [4,5].

The cellular constituents of the innate defense system in the intestine include epithelial cells, goblet cells, dendritic cells and macrophages. The front line of this system is the mucous layer containing goblet cells which secrete mucin [6,7]. The response of goblet cells was observed in several intestinal infections due to bacteria, viruses and parasites. Decreases number of goblets cells (hyperplasia) was observed in some parasitic infections like helminthes [8,9] but hypoplasia of these cells were observed in coccidial infection with Eimeria [10,11].

Due to infection with Plasmodium parasites, the host natural immune response is activated and generates large amounts of reactive oxygen species causing disorganization between oxidizing species and antioxidants [12]. This disorganization could lead to a status of oxidative stress. This induced oxidative stress is considered to be an important mechanism as a host response to infection that induces parasite death. Our study aimed to investigate the intestinal oxidative damage and mucin regulated gene response to *Plasmodium chabaudi* infection.

Materials and Methods

Animals

Twenty adult female C57Bl/6 mice weighed 23-27 g and aged 9-12 weeks were obtained from the animal facilities of King Saud University, Riyadh, Saudi Arabia. The mice were bred under specified pathogen-free conditions and

fed a standard diet and water *ad libitum*. The experiments were approved by state authorities and followed Saudi Arabian rules for animal protection.

Infection of Mice

Blood stages of *P. chabaudi* were weekly passaged in Swiss albino mice. Experimental animals were challenged with 10^6 *P. chabaudi*-parasitized erythrocytes. Parasitemia was evaluated in Giemsa stained blood smears, and total erythrocytes were counted in a Neubauer chamber.

Animals were divided into two groups. The first group served as a vehicle control. The second group was infected with 10^6 *P. chabaudi*-parasitized erythrocytes. All mice were sacrificed on day 8 post-infection.

Histological Analysis

Pieces of jejunum were freshly prepared from mice on day 8 postinfection with *P. chabaudi*, fixed in 10% neutral buffered formalin, and then embedded in paraffin. Sections were cut and then stained with hematoxylin and eosin. According to Dommels et al. [13], tissue sections were scored for inflammatory lesions (infiltrations by mononuclear cells, neutrophils, eosinophils, and plasmacytes, for fibrin exudation and lymphangiectasis, for tissue destruction (enterocyte loss, ballooning degeneration, edema, and mucosal atrophy), and for tissue repair (hyperplasia, angiogenesis, granulomas, and fibrosis). A rating score between 0 (no change from normal tissue) and 3 (lesions involved most areas and all the layers of the intestinal section including mucosa, muscle, and omental fat) was given for each aspect of inflammatory lesion, tissue destruction, and tissue repair. The sum of inflammatory lesions, tissue destruction, and tissue repair scores was used to represent the total histological injury score (HIS) for each intestinal section. The sum of the inflammatory lesions was multiplied by 2 to give more weight to this value since the tissue changes were mainly characterized by inflammatory lesions [13]. Stained tissue sections were imaged using light microscope (Olympus, Japan) provided with digital high resolution camera.

The number of Goblet Cells

Sections were stained with Alcian blue for determination of the goblet cells. For each animal, the number of goblet cells in the jejunum was counted on at least ten well-orientated villous-crypt units (VCU). Results were expressed as the mean number of goblet cells per ten VCU [14].

Oxidative Stress

Part of the jejunum was weighed and homogenized immediately in order to prepare a 50% (w/v) homogenate in an ice cold medium containing 50 mM Tris–HCl and 300 mM sucrose. The initial homogenate was centrifuged at 500 × g for 10 min at 4°C. The supernatant was diluted with the Tris sucrose buffer to give 10% and was then used for the various biochemical determinations.

Glutathione

Glutathione (GSH) was determined chemically in jejunal homogenate using Ellman's reagent [15]. The method is based on the reduction of Ellman's reagent (5,5dithiobis(2-nitrobenzoic acid) with GSH to produce a yellow compound. The chromogen is directly proportional to GSH concentration, and its absorbance was measured at 405 nm.

Lipid Peroxidation

Lipid peroxidation in jejunal homogenate were determined according to the method of Ohkawa et al. [16] by using 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid, followed by heating in a boiling water bath for 30 min. Thiobarbituric acid reactive substances were determined by the absorbance at 535 nm and expressed as malondialdehyde (MDA) equivalents formed.

Nitric Oxide

The assay of nitrite in jejunal homogenate was done according to the method of Berkels et al. [17]. In acid medium and in the presence of nitrite the formed nitrous acid diazotises sulphanilamide, which is coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish-purple color which was measured at 540 nm.

Quantitative Real-Time PCR

Trizol (Invitrogen) was used to isolate the total RNA from mice jejuna. DNA-freeTM kit (Applied Biosystem, Darmstadt, Germany) was used to digest the Contaminating genomic DNA. To synthesize cDNA, we used the QuantiTect® Reverse Transcription kit (Qiagen, Hilden, Germany). Real time PCR was performed in a Taq-Man7500 (Applied Biosystems) using the QuantiTect™ SYBR® Green PCR kit (Qiagen) and the gene-specific QuantiTectTM primer assay (Qiagen) according to the manufacturer's instructions. The primers for mucin genes (MUC2 and MUC4) and 18S rRNA were purchased from Qiagen (Hilden, Germany). Following an initial incubation at 50 ˚C for 2min, Taq polymerase was activated at 95 ˚C for 10 min, 45 cycles followed at 95 ˚C for 15 s, at 60 ˚C for 35 s, and for 30 s at 72 ˚C. PCR product was measured as SYBR green fluorescence at the end of the extension phase. All PCR reactions yielded only a single product of the expected size as revealed by melting point analysis and gel electrophoresis. Relative quantitative evaluation of amplification data was done using Taqman 7500 system software v.1.2.3f2 (AppliedBiosystems) and the $2^{-\Delta\Delta c}$ T method [18]. Expression of the genes was compared to 18S rRNA [19].

Statistical Analysis

Student's t-test was used to determine significant differences. Data were represented as means ± SD of triplicate experiments and $P \leq 0.05$ was used to denote statistical significance.

Results

The infection of female C57Bl/6 mice with 10^6 *P. chabaudi* parasitized erythrocytes induced a maximum parasitemia (about 47%) on day 8 p.i. Light microscopical inspection of hematoxylin-and-eosin-stained sections revealed that the epithelial cells of the jejunum of mice infected with *P. chabaudi* were injured (Figure 1). The injury was semi quantified by applying the scoring according to Dommels et al. [13]. The infection induced a marked inflammatory injury in the jejunum (Figure 2). In addition, examination of Alcian blue stained sections (Figure 3) showed a significant increase ($P \leq 0.05$) in goblet cell numbers seen at the jejuna villi (Figure 4).

On day 8 p.i. with *P. chabaudi* parasitized erythrocytes, there was a significant increase ($P \leq 0.05$) in the level of NO (539 ± 7, µmol/g) and MDA (28±1, nmol/g) (Table 1) while the level of GSH (7±0.1, mg/g) was significantly ($P \leq 0.05$) decreased by the infection (Table 1).

Quantitative real-time PCR was used to detect changes in the mRNA levels of mucin genes in the jejunum. Upon infection with *P. chabaudi*, there was a significant increase in the mRNA expression of MUC2 and MUC4 (Figure 5). Gene expression corresponding to MUC2, the main gel-forming secretory mucin in intestine, significantly ($P < 0.01$) increased in the jejunum of mice infected with *P. chabaudi* when compared to the non-infected animals (Figure 5). Also, MUC4, another membrane-associated mucin, was significantly ($P \leq 0.05$) up regulated by malaria infection (Figure 5). This upregulation was approximately two fold increase when compared to the non-infected control.

Discussion

Our previous studies illustrated that spleen and liver act as effectors against malaria infection [20, 21]. Up to date, there is no study investigated the role of the intestine against malaria infection except Chau et al. [22] who investigated that increasing L-arginine bioavailability via oral supplementation can ameliorate malaria-induced intestinal pathology.

Getting rid of plasmodial stages is mediated by both acquired [23] and innate [24] immune responses. The infected mice were able to heal the induced infection by *P. chabaudi* and also, develop immune response against reinfection. Our results is in agreements with other studies that found an increase in parasitemia during the phase of crises [25,26]..

Oxidative stress markers during infection are found in a significant increased level compared to the non-infected controls animals [27-29]. It is know that, oxidative stress could result from free radical production, this fact also may be due to increase malondialdehyde which is considere to be an important lipid peroxidation marker, this indicated that, oxidative stress is an important process during parasitic infection [30]. The high Also, the increased

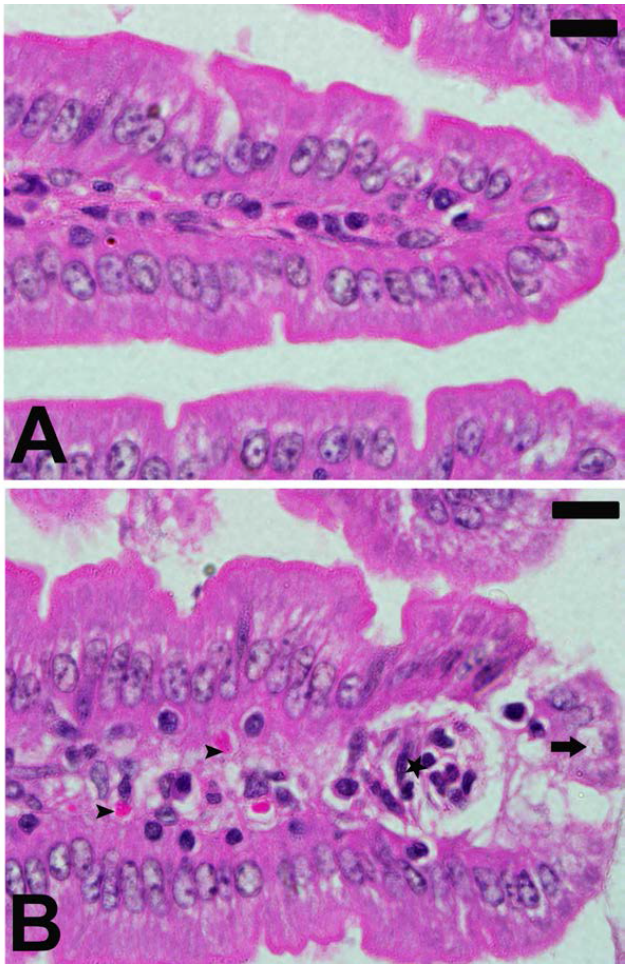

Figure 1. *Plasmodium chabaudi-induced changes in intestine histology of C57BL/6 mice. Section from non-infected group (A) and P. chabaudi infected group (B). Infection induced inflammation (astar) and cellular vacuolation (arrow). Also, some parasitized erythrocytes (arrow head) are present. Sections are stained with hematoxylin and eosin. Scales 25 µm.*

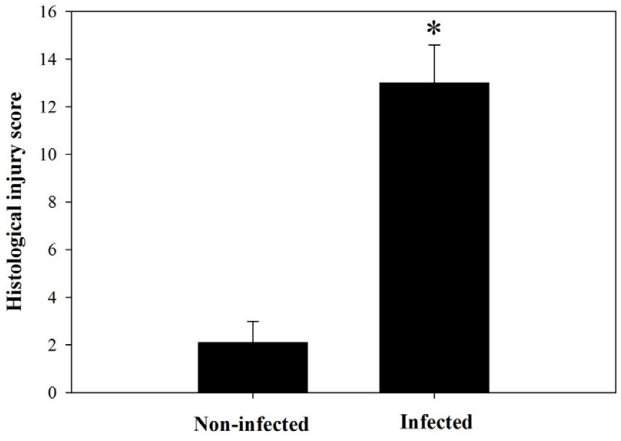

Figure 2. *Total histological injury scores in intestine of non-infected and infected mice with P. chabaudi on day 8 p.i. Scores were calculated according to Dommels et al. (2007). Values are means ± SD. *Significance against non-infected group at $p \leq 0.05$.*

level of TBARS, the lipid peroxidation product was seen in infected red blood cells by P. falciparum, P. vinckei, P. berghei, and P. chabaudi [31]. In addition, NO has an

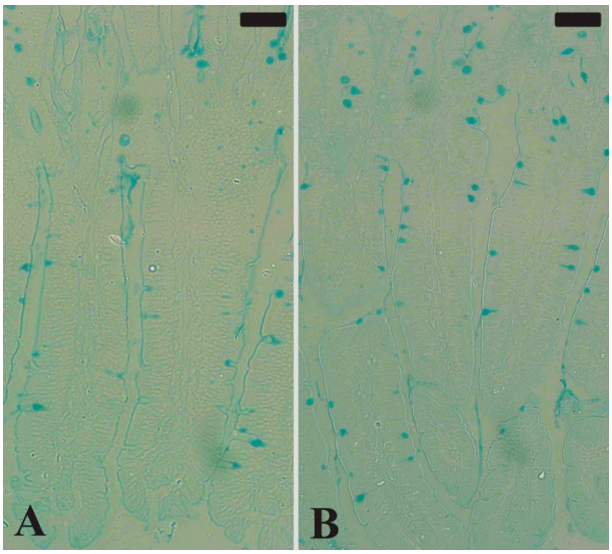

***Figure 3**. Goblet cells in mouse jejunum. Section from non-infected group **(A)** and P. chabaudi infected group **(B)**. Sections were stained with Alcian blue. Bar =50 µm.*

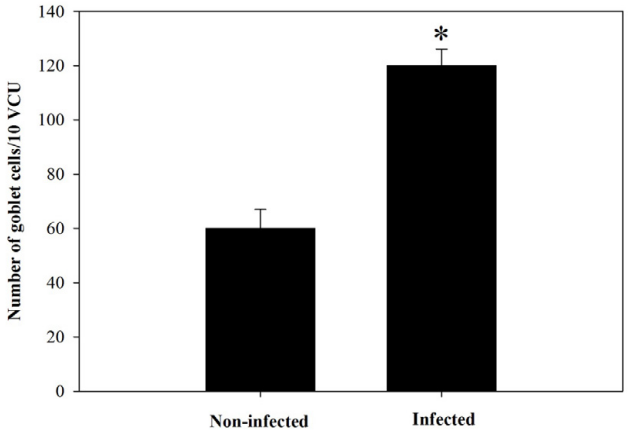

***Figure 4.** Goblet cell number in jejunum of non-infected and infected mice with P. chabaudi on day 8 p.i. Data were obtained from Alcian blue-stained sections as shown in Figure 3. Values are means ± SD. *Significance against non-infected group at $p \leq 0.05$.*

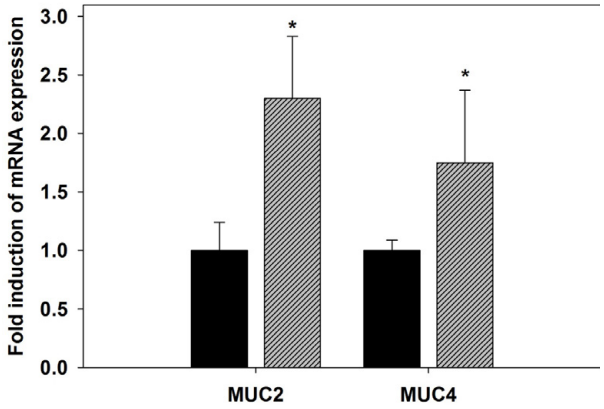

***Figure 5.** Quantitative RT-PCR analysis of MUC2 and MUC4 mRNA in the jejunum of mice. Expression was analyzed in non-infected and P. chabaudi infected mice on day 8 p.i., normalized to 18S rRNA signals, and relative expression is given as fold increase compared to the non-infected control mice. Values represent means ± SD. *Significance against non-infected male mice at $p \leq 0.05$.*

important role in malaria where increased serum levels of NO favor parasitemia resolution without affecting the host. Moreover NO was previously seen as a toxic agent which is also, responsible for the inflammatory processes and could indirectly activate cytokines to activate the immune system [12].

Hyperplasia of goblet cells due to malaria has not been described before. In our model, P. chabaudi infection is associated with increased goblet cells. However, hyperplasia of goblet cells has been investigated in a number of bacterial, viral and parasitic infections [32].

Moreover, our results of qRT-PCR, revealed that the expression of MUC-2 and MUC-4 were significantly increased due to P. chabaudi infection. It is documented that, MUC-2 is the first line of innate host defense that could act to prevent the induced injury due to infection [33]. In addition, Kim and Ho [33] reported that mucin plays a critical role in many stages of metastatic processes of colorectal cancer where colon cancer cell lines that studied for high capacity for metastasis showed to upregulate MUC2. Moreover, MUC4 plays important roles in the carcinogenesis and progression of multiple human cancers, including pancreatic cancer [34,35].

Collectively, studying the intestinal response to P. chabaudi infection could help in understanding the process of intestinal oxidative damage as well as the role of mucin related genes during infection. Further studies are required to know the mechanism and the pathway by which the parasite induce these intestinal alterations.

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