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The Effects of Iron Supplementation on the Growth Rate and Antioxidant Activity of *Trichomonas vaginalis*

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Abstract

Trichomonas vaginalis is a common protozoan parasite found in females and males worldwide. The parasite causes mild to severe inflammations in the urogenital tract. This study was conducted to evaluate the effect of iron excess on the growth rate and the activity of antioxidant enzymes in *T. vaginalis*. The parasite was cultured in Diamond's Trypticase Yeast Maltose (TYM) media with and without iron supplementation and assessed at 2, 3, 5, 7, 9, 11, 13 and 15 days post inoculation (dpi). The results showed significant higher numbers of the parasite in medium with iron supplementation. Furthermore, our findings revealed higher activities of antioxidant enzymes (SOD, GPX and CAT) in parasites cultured with iron supplementation. In conclusion, the present experiment showed that iron increased not only the multiplication rate but also the antioxidant activities of *T. vaginalis*. It seems that iron could protect *T. vaginalis* from toxic oxygen metabolites during tissue invasion and helps the parasite to maintain its pathogenicity for the host.

Keywords: *Trichomonas vaginalis*; Iron; Growth; Antioxidant enzymes

Introduction

Trichomonas vaginalis, a flagellated protozoan parasite, causes a prevalent sexually transmitted disease worldwide. The parasite mainly affects the urogenital tract of both men and women. In women, symptoms are heavier which range from mild to severe inflammation with a frothy malodorous discharge and severe irritation [1]. The parasite can invade the squamous epithelium and associates with vaginitis, low birth weights and many perinatal complications [2].

T. vaginalis lacks the ability to synthesize many of the macromolecules *de novo*. Therefore, the uptake of nutrients is from the vaginal secretions or through the host and/or bacterial cells [3]. This implicates that *T. vaginalis* is required to include many of the essential macromolecules in culture media. Among those molecules, vitamins and minerals such as iron seem very important for the survival of the parasite [4].

Iron is required to maintain maximal levels of ferredoxin and pyruvate-ferredoxinoxido reductase activity [5]. Also, it appears that resistance to complement-mediated lysis is dependent upon a high concentration of iron [6]. Iron has been demonstrated to upregulate the expression of cysteine proteinases, which have been found to degrade the C3 portion of complement on the surface of the organism which allows the organism to evade complement-mediated damage [6]. In addition, the pathogenesis of *T. vaginalis* is differentially modulated by iron [7]. Considering the role of iron for the pathogenesis *Trichomonas* in the host, it can be assumed that this element could have a significant effect on the growth rate of the parasite.

Free radicals are very reactive chemical substances which can cause oxidative damages to the living organisms. Under normal physiological conditions, there is a critical balance in the generation of free radicals and antioxidant defense systems against free radical injury [8,9]. The imbalance between free radical production and antioxidant defense creates a condition known as oxidative stress. Clinical observations and experimental evidence suggest that oxidative stress plays a dominant role in the host's defense against parasitic infections [10].

The function of antioxidant enzymes has to some extent been studied in ruminant hosts infected with the blood protozoan parasites [11-13]. The presence of superoxide dismutase (SOD) and NADH oxidases was reported in *T. vaginalis* [14,15]. However, the mechanisms of cellular protection against adverse effects of oxygen metabolites have not been investigated well in trichomonads. Therefore, this study was conducted in order to investigate the status of the iron supplementation on the growth rate and the activity of major antioxidant enzymes (glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) in *T. vaginalis*.

Materials and Methods

T. vaginalis cultivation

A *T. vaginalis* isolate, kindly obtained from Shiraz University of Medical Sciences, Shiraz, Iran was routinely cultured in Diamond's TYM medium [16], composed of 150 mg of Tryptone, 12 g of yeast extract, 5.5 g of glucose, 2.5 g of sodium chloride, 0.5 g of L-cysteine, 0.5 g of sodium thioglycolate, 80 mg of Gentamicin, 2 mg of Amphotericin B, Penicillin G (1 × 10^6 SI), 120 ml of horse serum and 0.75 g of agar. The medium was constituted per 1000 ml of distilled water and its pH was set at 6.2.

The parasites were recultured in TYM media with and without iron supplementation and kept at 37°C. Each group consisted of 48 microtubes containing 1.2 ml culture media and 10 µl media containing about 3000 *T. vaginalis* organism. In Iron-supplemented group, ferrous sulfate was also added to adjust a final Fe concentration on 400 µM [7]. Six microtubes from each group were removed and followed at 2, 3, 5,

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7, 9, 11, 13 and 15 days post inoculation (dpi). The cultured specimens were centrifuged at 3000 g for 3 min; the pellet was resuspended in 1 μL of sterile distilled water, shaken thoroughly and examined by a Neubauer cell chamber to calculate the parasite numbers per ml. Finally, the contents of each micro tube were kept at -20°C until the biochemical assays were performed.

Biochemical assays

Glutathione peroxidase (GPx) activity: The activity of GPx was evaluated with GPx detection kit (Ransel kit produced by Randox Co., UK) according to the manufacturer's instructions. GPx catalyze the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm against blank was measured spectrophotometrically. One unit (U) of GPx activity was defined as the amount of enzyme that converts 1 μmol of NADPH to NADP+ per minute. The GPx activity was expressed as unit per mg of protein (U/mg protein).

Superoxide dismutase (SOD) activity: SOD detection kit (Ransod®, Randox Co., UK) was used to evaluate total SOD activity. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes 50% inhibition of the rate of reduction of INT under the conditions of the assay. SOD levels were recorded at 505 nm and through a standard curve, and expressed as unit per mg of protein (U/mg protein).

Catalase (CAT) activity: Tissue catalase activity was assayed spectrophotometrically by monitoring the decomposition of H_2O using the procedure of Aebi [17]. Briefly, 0.5 ml of 30 mmol/l H_2O solution in 50 mmol/l phosphate buffer (pH=7.0) and 1 ml of 1:10 diluted tissue supernatant was added and the consumption of H_2O was followed spectrophotometrically at 240 nm for 2 min at 25°C. The molar extinction coefficient was 43.6 l/mol per cm for H_2O. Catalase activity was expressed as the unit that is defined as μmol H_2O consumed/min per mg of protein. In order to achieve the average activity of each enzyme in the whole parasite population, the values measured for each case were divided into the parasite number.

Statistical analysis

Student's *t* test was used to compare the effects of iron supplementation on the evaluated parameters. All data were analyzed using the Statistical Package for Social Sciences (SPSS, 16.0) and the significance was set at P<0.05.

Results

The pattern of alterations in the parasite number of both culture media is presented in Figure 1. Our data showed that although the parasites number was increased in both media from the start of the study toward the end, it was overall higher in iron-rich medium. Significant elevations of parasite number were seen at 2, 5, 7 and 15 days post inoculation (dpi) in iron-rich medium compared to usual Diamond's TYM medium. The alterations in the activity of antioxidant enzymes in different days are presented in Figure 2. The results revealed that the activity of GPx was significantly higher in parasites cultured in iron-rich medium. These differences were recorded at 2, 3, 5, 7, 11, 13

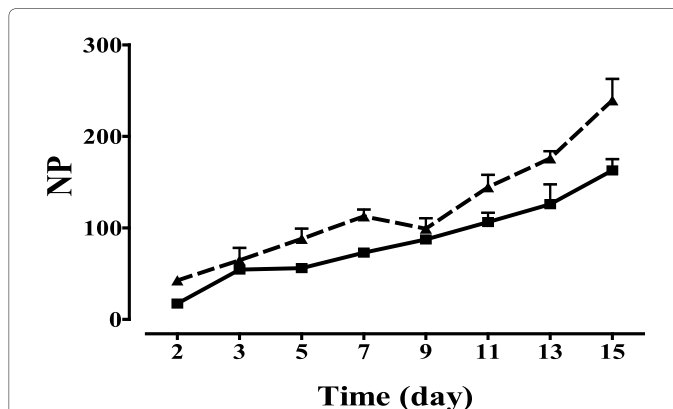

Figure 1: Number of *T. vaginalis* cultured in Diamond's Trypticase Yeast Maltose (TYM) media with (▲) and without iron supplementation (■) during 15 days post inoculation. NP: Number of Parasites ((organism/ml) × 10000).

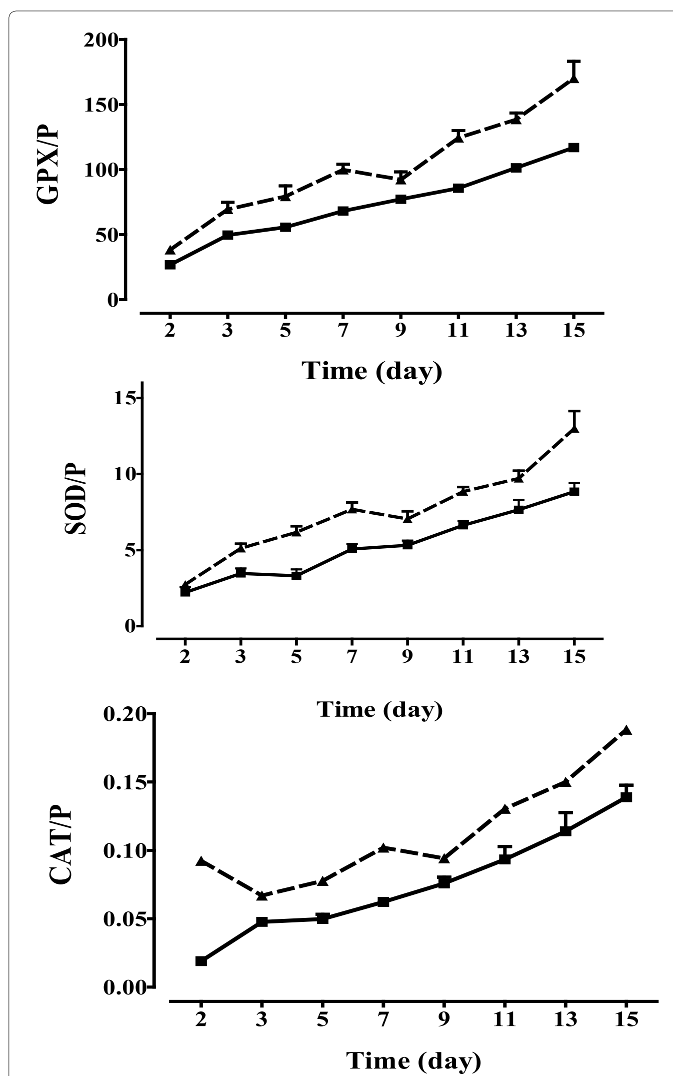

Figure 2: The activity of antioxidant enzymes (as unit/mg) in *T. vaginalis* cultured in Diamond's Trypticase Yeast Maltose (TYM) media with (▲) and without iron supplementation (■) during 15 days post inoculation. Note that the average activity of each enzyme in the whole parasite population was divided into the parasite number.

and 15 dpi; however, no significant difference was observed at 9 dpi.

A remarkable increase in GPx activity was evident in *T. vaginalis* cultured in iron-rich condition at 2, 3, 5, 7, 11, 13 and 15 dpi, but no significant difference was recorded at 9 dpi. Remarkable elevations were also observed in SOD activity in *T. vaginalis* grown in iron-rich medium at all samplings except for 2 dpi. The pattern of changes in the activity of Catalase (CAT) was different from that of GPx. According to our data, a significant increase in CAT activity was evident at 5, 7, 11, 13 and 15 dpi but not at 2, 3 and 9 dpi.

Discussion

In the present study, the growth rate of *T. vaginalis* grown in the presence of iron was significantly higher compared to those cultured in medium with no iron supplementation. These observations reinforce the idea that increasing the level of iron favors the multiplication of the parasite, thus exacerbating the symptoms of the disease. Similarly, previous reports confirmed that *T. vaginalis* essentially requires iron for its growth in the human vagina, where the iron concentration is changing. This element could increase the parasite multiplication in different culture media [5,18]. It seems that iron is an important nutrient for the growth of different protozoan genera such as *Entamoeba*, *Leishmania*, *Plasmodium* and Trichomonads. *Leishmania* and *Plasmodium* can use haemoglobin as a source of iron by catabolism of the haem group [19]. *Leishmania chagasi* also expresses a NADPH-dependent iron reductase capable of converting oxidized Fe^{3+} into the more soluble Fe^{2+} form [20]. In addition, the ability of *Tritrichomonas foetus* for the uptake of iron from the host environment may be a critical factor for its pathogenicity and virulence [21]. The parasite grows in the bovine vagina where lactoferrin is available and also invades the bovine uterus, where abundant transferrin exists [22].

Corroborating our data, Lehker and Alderete [18] assessed the responses of *T. vaginalis* to iron depletion or iron excess to evaluate the regulatory role of this element on certain properties of the parasite. They stated that, in comparison with organisms grown in excess iron, iron restriction resulted in greater than or equal to 80% lower rates of protein synthesis, greater than or equal to 3-fold decreases in cell densities and 2.5-fold longer generation time.

The role of iron in *T. vaginalis* has been shown to be associated with the virulence of the parasite in mice [7]. In addition to the increased virulence, iron also enhances the level of adherence and the cytotoxicity of Trichomonads [7].

High metabolic rate of the rapidly growing and multiplying parasites will produce large amounts of toxic by-products in the cells. Excessive *in vivo* generation of these products can adversely affect the cell functioning. The accumulation of high concentrations of free radical, mainly the reactive oxygen species (ROS), could damage the cell phospholipid membranes and vital macromolecules [23]. This condition is considered as oxidative stress which is indicated by impairment of antioxidant systems [24]. The balance between free radicals and antioxidants may be disrupted in many diseases. Previous studies show that protozoan parasite infections such as *Plasmodium* sp. [25], *Trypanosoma* sp. [26], *Trichomonas foetus* [27] and some nematodes [28,29] can induce oxidative stress in the host. However, defense mechanisms operating against reactive metabolites of molecular oxygen in *T. vaginalis* have not been studied in detail.

The results obtained here corroborate that iron has significant effect on the antioxidant activity of *T. vaginalis in vitro*. We revealed that the activities of GPx, SOD and CAT were significantly higher in iron-supplemented media. Higher numbers of parasites along with the increased amounts of antioxidant activities in the parasites grown in the presence of iron clearly indicate that this element enhances the strength of *T. vaginalis* to resist the vaginal defense mechanisms. Corroborating our data, Razavi et al. [30] studied the status of the activity of antioxidant enzymes in *Trichomonas gallinae* cultured in aerobic and anaerobic media. They revealed higher activities of GPx, SOD and CAT activities in *Trichomonas* cultured in both environments. These conditions stimulate the production of those protective enzymes at early and late stages of cultivation, depending upon the presence of oxygen in culture media. In contrast to these results, Ellis et al. [31] stated that *T. vaginalis* trophozoites lack the major peroxide-reducing enzymes, catalase and glutathione peroxidase. However, the presence of glutathione reductase and SOD activity suggested that some detoxification mechanisms may operate in *T. vaginalis.* This controversy is likely due to the sensitivity of *T. vaginalis* to oxygen above physiological levels which led to the lack of adequate peroxide reducing enzymes.

Antioxidant defense mechanisms have been indicated in other protozoans. Incubation of *E. histolytica* isolates under different culture conditions with an oxygen radical generating system induces an increase in SOD activity [32]. Therefore, it was suggested that regulation of SOD may support the parasite from invasion by oxygen metabolites. Although the specific activity of FeSOD in *E. histolytica* is reduced in the presence of a ferrous iron chelator (1, 10-phenanthroline), the total activity was found to be increased substantially. It was hypothesised that at low Fe^{2+}-levels in *E. histolytica*, the iron molecule in FeSOD may be replaced by an alternative divalent cation, like Mn^{2+}. Also, it can be argued that the affinity of iron to the enzyme is much higher than to the repressor molecules.

In conclusion, it can be noted that iron-supplemented media stimulate not only the higher growth rates but also the higher activity of antioxidant enzymes (SOD, GPX and CAT) in different days post inoculation for *T. vaginalis.* These observations could suggest that positive regulations of antioxidant enzymes in the presence of iron may contribute to protect *T. vaginalis* from oxygen free radicals during tissue invasions.

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Conflict of Interest

The authors declare no conflicts of interest.

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