Kerman University of Medical Sciences and Health Services
Scientific Research Journal
Journal of Kerman University of Medical Sciences
JKMU
Journal of Kerman University of Medical Sciences, 2020; 27 (3): 222-231

Study of Antioxidant Effects of Selenium-Enriched *Saccharomyces* Boulardii on *Staphylococcus* Aureus Infection

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Received: 13 July, 2019 Accepted: 26 April, 2020

ARTICLE INFO

Article type:
Original Article

Keywords:
Saccharomyces boulardii
Selenium
Oxidative stress
Staphylococcus aureus
Rat

Abstract

Background: The most important strategy of the immune system against pathogens is producing active oxygen intermediates with sidelong consequence of oxidative stress induction in body. Probiotics and selenium have recently been proven to be powerful antioxidants that help boost the immune system. Hence, the aim of this study was to investigate the antioxidant effects of *Saccharomyces boulardii* and selenium-enriched *S. boulardii* against oxidative stress induction caused by *S. aureus* in rats.

Methods: A total of 36 rats were divided into 6 groups. A: control group, B: *S. boulardii* treated group, C: selenium-enriched *S. boulardii* treated group, D: *S. aureus* infected group, E: *S. aureus* infected and *S. boulardii* treated group and F: *S. aureus* infected and selenium-enriched *S. boulardii treated* group. At the end of the treatment period, animals were anesthetized and blood samples were collected to measure blood cell count, their indexes and antioxidant factors.

Results: There was no significant difference in red blood cell count and its indexes, hematocrit percentage, hemoglobin concentration and also platelet count among experimental groups. While a significant increase in the number of white blood cells was observed in D and E groups compared to control group, the changes in other groups were not significant. Total antioxidant capacity, glutathione concentration and glutathione peroxidase activity decreased in D and E groups, compared with control group. Decreased glutathione peroxidase activity was significant only in F compared to the control group.

Conclusion: it is suggested that *S. boulardii* in selenium-enriched status has strong antioxidant effect against oxidative stress caused by *S. aureus* infection.

Introduction

S. aureus is one of the most important pathogens causing food poisoning and infecting hundreds of thousands of people every year (1). This bacterium is found on mucosal membranes and skin of mammals as well as in various nutrients and environments and is the cause of pneumonia, bovine mastitis, veins inflammation, meningitis, urinary tract infection, local inflammation of bones, endocarditis, skin surface lesions, and so on (2).

After pathogen attack and during early stages of infection, the phagocyte cells including neutrophils and macrophages migrate to infection site and produce superoxide anions (such as hypochlorous acid and nitric acid) to phagocyte foreign agents. These anions also react with other molecules and form various reactive oxygen species (ROS), such as hydrogen peroxide, proxynitrite and hypochlorite which cause oxidative stress induction in body (3).

Oxidative stress is an imbalance between the oxidant / antioxidant status of body which is resulted from attenuation of antioxidant defense system and exposes the body to a variety of diseases (4). Many natural compounds have been identified that have antioxidant properties and are able to strengthen the antioxidant system of body and subsequently reinforce the immune system. Flavonoid metabolites from plant sources, vitamin C and selenium are some of the examples of antioxidant factors (5). Selenium, as a potent antioxidant, plays an important role in intracellular reduction–oxidation reactions. This element is one of the components of selenoproteins and important enzymes in body, such as glutathione peroxidase and iodothyronine 5-deiodinase (6). Antioxidant function of selenium through some of the selenoproteins directly inhibit oxidative stress (7).

Recently, it has been reported that probiotics have antioxidant properties through producing various vitamins specially vitamin C and B complex vitamins, removing metal ions, inhibiting production of oxygen free radicals and oxidant compounds (8). Furthermore , these microorganisms, without launching harmful inflammatory responses, are able to cope with pathogenic bacteria via increasing anti-inflammatory cytokines and immunoglobulin levels, increasing proliferation of mononuclear cells and activating macrophages (9,10). Probiotics are alive microorganisms sufficient amounts of which are beneficial for health of the host. Some types of bacteria, in particular, lactic acid bacteria and bifidobacteria and *S. boulardii*, as a yeast, can be named as the most important probiotics (11,12).

Therefore, the purpose of present study was to explore the effects of selenium-enriched *S. boulardii* on oxidative stress induced by *S. aureus*.

Materials and Methods

S. boulardii enrichment with selenium

S. boulardii strain which is marketed as Yomogi capsule was purchased from Ardeypharm Company and a standard suspension was prepared. In order to enrich *S. boulardii* yeast, at first, the antibacterial effects of the three concentrations of selenium (0.001, 0.005, 0.01) were evaluated by macrodilution and the minimum inhibitory concentration (MIC) 0.005mg/ml was considered. Then, 90 µl sodium selenite (0.005 mg/ml) was added to 100 ml yeast suspension and it was incubated at 37°C for 48 hours (13). To remove excess selenium, suspension was centrifuged at 3000 RPM for 15 minutes and pellet was rinsed with normal sterile saline solution twice. Then, a suspension with concentration of 0.5×10^8 CFU/ml(0.5 McFarland) was prepared from the yeast pellet by spectrophotometer (13).

At the end of treatment period, rats were anesthetized and blood samples were directly collected from heart. Blood cell number and its indexes were measured using cell counter system (Mindray BC 6800 model). Total antioxidant capacity (T-AOC) was evaluated by T-AOC colorimetric assay kit (EASTBIOPHARM company) using ELISA plate reader. Glutathione peroxidase activity (GPX) and glutathione concentration (GSH) were assessed in 412 nm wavelength according to kit protocol prepared from ZellBio GmbH Company.

Animal care and grouping

A total of 36 adult female Wistar rats (150-220g) were prepared from Islamic Azad University of Falavarjan branch and kept in standard environmental conditions, with

temperature of 20-25˚C, humidity of 50±5% and 12:12 h light-dark cycle. Food and water were provided *ad libitum*. Rats were divided into 6 groups as follows:

A- Control group: This group was only fed with enough food and water for 4 weeks. In order to exert stress of gavage and injection, every other day to the end of examination, 1 ml of physiological serum was intragastrically given to rats and at the end of the second week, 0.5 ml of physiological serum was injected into peritoneum cavity of rats.

B- *S. boulardii* treated group: This group intragastrically received 1 ml of yeast suspension (0.5×10^8 CFU/ml), every other day for 4 weeks and at the end of the second week, an intraperitoneal injection of 0.5 ml of physiological serum was made.

C- Selenium-enriched *S. boulardii* treated group: This group was administered 1 ml of *S. boulardii* suspension enriched with selenium (0.5×10^8 CFU/ml) in quite similar conditions to the previous group.

D- *S. aureus* infected and *S. boulardii* treated group: In this group, similar to group B, rats were administered yeast suspension, with the difference that, at the end of the second week, this group received an intraperitoneal injection of 0.5 ml of *S. aureus* (ATCC 25943) suspension (1.5×10^8 CFU/ml).

E- *S. aureus* infected and selenium-enriched *S. boulardii* treated group: In this group, rats were given selenium-enriched *S. boulardii* similar to group C and at the end of the second week, an intraperitoneal injection of 0.5 ml of *S. aureus* suspension was made.

F- *S. aureus* infected group: this group was treated with adequate food and water for 4 weeks and every other day, rats were intragastrically given 1 cc of physiological serum. At the end of the second week, 0.5 ml of *S. aureus* suspension with concentration of 1.5×10^8 CFU/ml was injected into peritoneum cavity of rats.

Ethical Considerations

The permission for the use of animal samples in the experiments was taken from the institutional review board of the Islamic Azad University of Flavarjan Branch (No: IR.IAU.FALA.REC.1396.011), after considering the project and its aims.

Statistical analysis

The data are presented as mean ± standard deviation. Using SPSS software (v. 21) and one-way analysis of variance (one-way ANOVA) test, the statistical analysis was performed. A substantial difference was based on eventuality of less than 0.05 ($P<0.05$).

Results

As presented in Table 1, WBC count significantly increased in two groups of *S. aureus* infected- *S. boulardii* treated and *S. aureus* infected rats as $p\leq0.05$ and $p\leq0.01$, respectively. Significant decrease in the number of these cells was observed in control group, *S. boulardii* treated group and *S. aureus* infected- selenium-enriched *S. boulardii* treated group, compared to *S. aureus* infected group ($p\leq0.01$). The data demonstrated no significant difference in RBC count, MCH and MCHC indexes, hemoglobin concentration, platelet count and hematocrit percentage between treatment groups and control group.

Table1. Evaluation of hematological parameters in experimental groups

hematological parameters	Experimental groups					
	Control	S. *boulardii*	Se-Enriched, S. *boulardii*	S. *aureus*, S. *boulardii*	S. *aureus*, Se-Enriched, S. *boulardii*	S. *aureus*
WBC(10^3/ µl)	##5.90±1.356	##6.07±1.078	7.02±1.548	9.12±1.161*	##6.15±0.987	9.73±2.714**
RBC(10^6/ µl)	7.49±0.380	7.231±0.313	7.52±0.97	7.34±0.479	6.89±1.034	7.34±1.249
HGB(g/dl)	14.36±0.637	13.92±0.584	14.57±0.947	14.17±0.631	14.23±0.850	14.56±0.811
HCT (%)	43.92±1.586	41.70±2.441	42.50±2.551	42.68±2.576	43.33±3.212	45.05±2.755
MCV(fl)	58.65±1.049	57.65±1.098	56.78±1.126	58.10±1.171	58.20±1.645	58.46±1.11
MCH(pg)	19.18±0.953	19.23±0.455	18.23±0.763	19.30±0.622	18.42±0.534	18.85±0.225
MCHC(g/dl)	32.40±0.704	33.25±0.677	32.08±0.98	32.88±0.483	31.66±0.864	32.21±0.722
PLT (10^5/ µl)	8.71±1.269	8.65±0.700	9.53±0.588	8.55±0.625	8.91±0.933	8.53±0.874

# indicates significant difference compared to pathogen infected group (#p≤0.05, ##p≤0.01). * indicates significant difference compared to control group (*p≤0.05, **p≤0.01). Data are presented as Mean ± SD.

GSH concentration decreased in *S. aureus* infected- *S. boulardii* treated and *S. aureus* infected groups in comparison to control group with significance level of p≤0.01. Despite the reduced concentration of GSH in *S. aureus* infected- selenium-enriched *S. boulardii* treated group, the decreased amount was not significant compared to control group. GSH concentration was significantly higher in control (p≤0.01), yeast treated (p≤0.05) and selenium-enriched yeast treated (p≤0.001) groups, compared with *S. aureus* infected group. Changes of GSH level in two groups of pathogen infected- yeast treated and pathogen infected- selenium-enriched yeast treated rats were not significant compared to *S. aureus* infected rats. (Figure1)

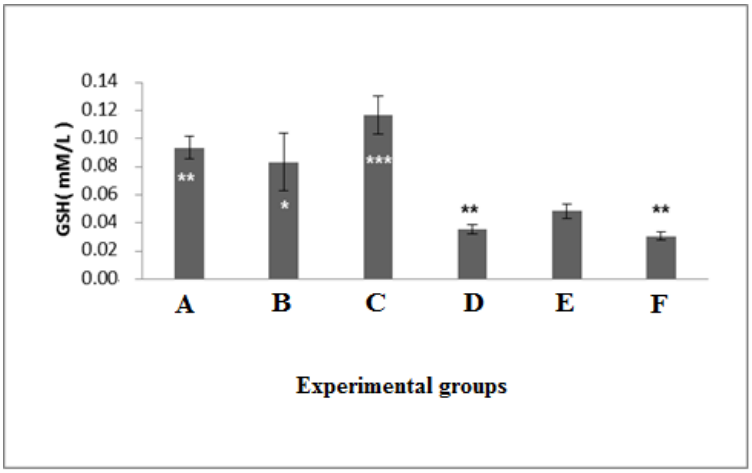

Figure 1. Results of glutathione concentration measurement. Asterisks above the error bar indicate significant difference compared to control group and asterisks under the error bar indicate significant difference compared to pathogen infected group (*p≤0.05,**p≤0.01, ***p≤0.001). Data are presented as Mean ± SD. A: Control, B: S. boulardii treated group, C: Selenium-enriched S. boulardii treated group, D: S. aureus infected and S. boulardii treated group, E: S. aureus infected and selenium-enriched S. boulardii treated group, F: S. aureus infected group

GPX activity significantly decreased in three groups of *S. aureus* infected rats, *i.e.* pathogen infection alone, in combination with *S. boulardii* treatment and in combination with selenium-enriched *S. boulardii* treatment in comparison with control group (respectively p≤0.001, p≤0.01, p≤0.05). A significant increase in GPX activity was observed in selenium-enriched *S. boulardii* treated rats compared to control group (p ≤0.01). Three groups of uninfected rats showed higher enzyme activity (p≤0.001) than pathogen infected group (Figure2). T-AOC decreased in rats of *S. aureus* infected (p≤0.001) and *S. aureus* infected- *S. boulardii* treated (p≤0.05) groups and observed changes in other groups were not significant in comparison to control group. Higher levels of T-AOC were observed in four groups of control, *S. boulardii* treated, selenium-enriched *S. boulardii* treated and *S. aureus* infected- selenium-enriched *S. boulardii* treated rats in comparison to *S. aureus* infected group (respectively, p≤0.001, p≤0.05, p≤0.001 and p≤0.001). (Figure3)

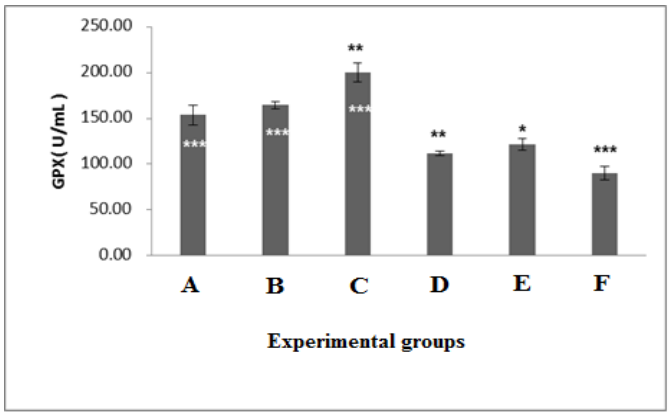

Figure 2. Results of glutathione peroxidase activity measurement. Asterisks above the error bar indicate significant difference compared to control group and asterisks under the error bar indicate significant difference compared to pathogen infected group (*p≤0.05,**p≤0.01, ***p≤0.001). Data are presented as Mean ± SD. Control, B: S. boulardii treated group, C: Selenium-enriched S. boulardii treated group, D: S. aureus infected and S. boulardii treated group, E: S. aureus infected and selenium-enriched S. boulardii treated group, F: S. aureus infected group

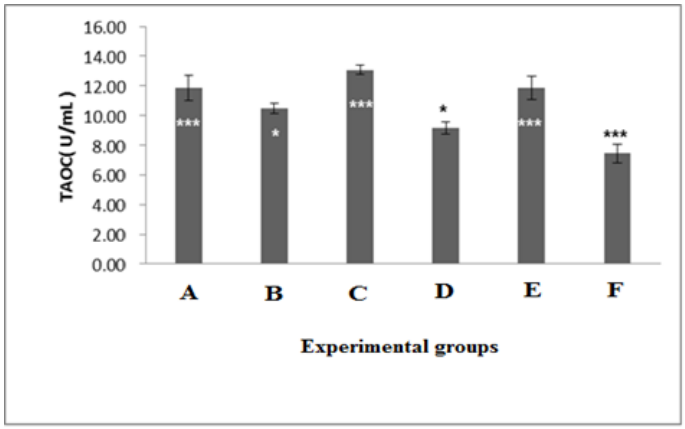

Figure 3. Results of total antioxidant capacity measurement. Asterisks above the error bar indicate significant difference compared to control group and asterisks under the error bar indicate significant difference compared to pathogen infected group (*p≤0.05,**p≤0.01, ***p≤0.001). Data are presented as Mean ± SD. Control, B: S. boulardii treated group, C: Selenium-enriched S. boulardii treated group, D: S. aureus infected and S. boulardii treated group, E: S. aureus infected and selenium-enriched S. boulardii treated group, F: S. aureus infected group

Discussion

A number of *in vitro* studies have shown that pathogens are able to bind to RBCs through the mediation of plasma proteins (such as fibrinogen and IgG) and can change the number of RBCs and their indexes (14). Platelets are also able to attach to bacteria and affect both innate and adaptive immune responses through interactions with various types of leukocytes (15).

In the present study, hematological parameters were compared among experimental groups. RBC count and its indexes, platelet number, hematocrit percentage and hemoglobin concentration did not show any significant changes in test groups in comparison with control group. Higher amounts of WBC were observed in two groups of *S. aureus* infected and pathogen infected- yeast treated rats. WBC increase showed a higher significance level in *S. aureus* infected group. Ren *et al.* reported a rise in WBC number in mice following *Salmonella* infection, and augmentation of the host defense response was stated as the consequence of pathogen entry (16).

Fasulkov *et al.* observed substantial leukocytosis after inducing *S. aureus* mastitis in Bulgarian local goats (17). Also in this study, higher level of WBC in *S. aureus* infected rats was due to stimulation of the immune system and accumulation of WBCs in blood. Despite higher WBC count in *S. aureus* infected rats which were treated with *S. boulardii*, the increased amount was less than in *S. aureus* infected group, probably because of *S. boulardii* antioxidant properties, which moderated oxidative stress arising from *S. aureus* infection by boosting antioxidant system of body. It has been reported that probiotics are able to remove metal ions, inhibit oxygen free radicals and oxidant compounds and prevent their production by antioxidant mechanisms and all of these help strengthen immune system (8). These compounds inhibit lipid peroxidation, protein oxidation and cellular DNA destruction and cause subsequent resistance to infections, inflammation and chronic diseases (18).

WBC level did not show any significant difference between *S. aureus* infected-selenium enriched yeast group and control group. It is likely that selenium, which acts as a potent antioxidant, in synergy with *S. boulardii*, increases body resistance of rats to pathogens and prevents pathological stimulation of the immune system (6).

Selenium is a powerful nutritional antioxidant which carries biological effects through its integration with selenoproteins. Considering the crucial role that selenoproteins play in regulating ROS and redox conditions adjacent to all tissues, selenium supplements strongly affect inflammation and immune responses and improve immune system functions (19). Boostani *et al.* reported the ability of supplements containing selenium in resolving oxidative stress, followed by inflammation alleviation and immune system adjustment (20).

In the current study, blood T-AOC was compared among groups. Although T-AOC level reduction was significant in pathogen infected- yeast treated group, there was no significant difference between T-AOC of infected- selenium-enriched yeast treated rats and the control group. Ghasemian and Salimi-Bejestani observed a sharp decline in T-AOC amount after inducing *S. aureus* mastitis in cows (21).

Principal mechanism of immune cells against pathogens entering the body is elevation of active oxygen intermediates such as H_2O_2, NO and increasing body oxidative stress. In this circumstances antioxidant defense system of the body tries to compensate the effects, but sometimes, it is not able to balance the oxidant / antioxidant status, hence antioxidant factors level

decline (22). Based on the findings of the present study, it seems that *S. aureus* induced-oxidative stress has weakened antioxidant system. Under such conditions, externally supplied antioxidant can balance the oxidative status of the body.

The results showed that *S. boulardii*, alone, was able to increase antioxidant level of body to some extent, but as enriched with selenium, it enhanced T-AOC to control level. It seems that this probiotic and selenium are able to adjust increased level of oxidative stress through antioxidant properties. There are extensive studies revealing positive effects of probiotics and selenium on improvement of antioxidant system. It has been reported that using supplements of selenium-enriched *S. boulardii* enhances fertility in rats and the presence of antioxidant compounds in supplement was named as the main factor effective on fertility rate increase (23). Chen *et al.* added selenium-enriched *S. boulardii* to food of Arbor Acres broilers and observed improved function of antioxidant enzymes in liver of chickens (24). Another study showed antioxidant supplements especially selenium compounds and vitamin E reduced production of free radicals and augmented power of enzymatic antioxidant defense system (25). Amaretti *et al.* reported that probiotics are able to remove metal ions, inhibit oxygen free radicals and oxidant compounds and prevent their production by antioxidant mechanisms (8).

Non-significant change in T-AOC level of the two groups treated with *S. boulardii* and selenium-enriched *S. boulardii*, in comparison to control group, revealed that in normal situations that endogenous antioxidants have maintained the balance between oxidant/antioxidant statuses of body, exogenous antioxidants have no side effects on body and consumption of them is likely to be safe. The first defense line of antioxidant system to deal with oxidative stress is converting glutathione to its oxidized form, which is done by GPX. Accordingly, we aimed to examine activity level of GPX and GSH concentration in assay groups. In this study, GSH concentration and GPX activity significantly decreased in two groups of *S. aureus* infected rats, *i.e.* treated and non-treated with *S. boulardii* compared to control group. A recent study has shown that *S. aureus*-induced oxidative stress lessens reduced form of glutathione (GSH), GPX, glutathione reductase and activity of glutathione s-transferase, with extending time of vancomycin-sensitive *S. aureus* infection in murine peritoneal macrophages, but oxidized glutathione amount significantly increased (26). Following *S. aureus*-induced hepatotoxicity in rats, significant increase in lipids peroxidation along with reduction in GPX, glutathione reductase, glutathione s-transferase and also glutathione concentration were observed in a research conducted by Prasad *et al.* (27). It has been reported that infecting animals with *Pseudomonas aeruginosa* results in increased lipid peroxidation and less amount of GSH which are associated with, lung injury indicators, neutrophils infiltration and weakened antioxidant defense. These changes are accompanied by a reduction in GPX activity (28).

Along with the results of the previous investigations, GPX and GSH level reduction in current study, following *S. aureus* infection, confirmed the weakness of antioxidant system. In comparison to infected group, there are no significant changes in GPX and GSH levels of the two groups of infected-yeast/selenium-enriched yeast treated rats. On the other hand, by comparing these two pathogen-infected treated groups with control group, reductions in GPX and GSH levels were observed. *S. boulardii* treatment on infected group, was not able to raise antioxidant level but as enriched with selenium, increased the amount of the two mentioned factors. It means,

selenium-enriched yeast is able to alleviate harmful effects of pathogen on antioxidant system to some extent and not very intense.

The results of this study suggest that selenium is the main agent affecting GSH level. As a cofactor, selenium plays a role in so many enzymes, for instance GPX, and through this, selenium protects the cell against oxidation by free radicals (29,30). In other words, selenium is an inseparable part of the catalytic site of GPX and participation in selenoproteins formation is identified as the function of this cofactor (31).

GPX activity significantly increased in uninfected-selenium-enriched *S. boulardii* treated group, as well as GSH concentration, though not significantly. This can affirm the claim that selenium as an antioxidant plays an outstanding role in strengthening agents of body antioxidant system, especially GPX (32).

Conclusion

The findings of the present study showed that inducing immune system by *S. aureus* resulted in an increased number of WBC and protective function of these cells led to an increased oxidative stress in body. *S. boulardii*, especially in status of being enriched with selenium as an antioxidant, was able to somewhat reinforce antioxidant system of body and without side effects on RBC number and its indexes, prevent excessive oxidative stress levels following *S. aureus* infection.

Acknowledgments

This article is part of a microbiology thesis in Islamic Azad University, Flavarjan Branch. We appreciate the University for providing us necessary equipment to do this scientific research.

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