Lipid peroxidation and antioxidant status in vagina microenvironment of patients with several common vaginitis

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Summary

Objective: Oxidative stress has been suggested to play an important role in many diseases, including vaginitis. To evaluate oxidative biomarkers in the secretion of cervix samples of vaginitis, this study will illustrate the status of lipid peroxidation and antioxidant status in vaginal microenvironment. *Materials and Methods:* A total of 257 patients with vaginitis, including candida vaginitis, bacterial vaginosis, and trichomonas vaginitis were involved in this study. Cervico-vaginal fluid was collected from these patients before and after treatment, and the malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), hydrogen peroxide (H_2O_2), and vitamin C levels were measured by enzyme-linked immunosorbent assay (ELISA). *Results:* The results revealed that the MDA and H_2O_2 levels were increased in the vaginitis patients, while there was no significant difference in MDA level among different kinds of vaginitis before treatment. The CAT and vitamin C levels in vaginitis were decreased before treatment. Moreover, the data also showed that the MDA and H_2O_2 levels were decreased, while the CAT, SOD, and vitamin C levels were increased after received treatment, respectively, and there was no significant difference between controls and vaginitis. *Conclusion:* This study indicated that oxidative stress played an important role in vaginitis.

Key words: Vaginitis; Oxidative stress; Microenvironment; Biomarker.

Introduction

Vaginitis is a common disease caused by an infection or by non-infectious causes. In women of childbearing age, the incidence of bacterial vaginosis was 40%-50%; while the incidence colpitis mycotica and trichomonas vaginitis were 20%-25% and 10%-15%, respectively [1-3]. Vaginitis is prone to relapse because its mechanism is still unknown [4-5]. Previous studies had demonstrated that the alteration, exudation, tissue damage, oxidative stress, etc might be involved in the infection and inflammation.

Oxidative stress is defined in general as an excess formation and insufficient removal of highly-reactive molecules, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [6, 7]. Free radicals are continually produced in the body as a result of normal metabolic process and interaction with environmental stimuli. Under physiological conditions, a wide range of antioxidant defenses protect against the reduced activity of antioxidant defenses or both of these phenomena result in oxidative stress. Some major antioxidant enzymes are superoxide dismutase (SOD) and catalase (CAT). Malondialdehyde (MDA), which plays important roles in antioxidization damage, helps the animal to cope with oxidative damage [8, 9]. Free radicals under the function of SOD would produce oxygen molecule and hydrogen peroxide (H_2O_2). H_2O_2 would be converted to water under the function of catalase, so as to clean free radicals to reduce lipidic superoxide damage. Excess stress response would inhibit immunity function, causing physiological dysfunction, increasing susceptibility to infection, and even death. The origin of stress includes variation in temperature, infection, shock, etc. Therefore, how to prevent stress response and alleviate the harm caused by stress is currently one of the key subjects of research in this field. However, oxidative stress is not an easily definable condition, and none of the indices used for its evaluation could be defined as the most appropriate criteria in universal terms.

As one kind of infection, vaginitis may be related to oxidative stress, and the ROS and antioxidant enzymes participate in the progress of vaginitis. However, the exact biological role of oxidative stress and antioxidants in patients with vaginitis remains so far equivocal. Besides, the level of oxidative stress in the vagina of the patients with local vaginitis is still unclear.

Mougeot *et al.* found that oxidative stress in the pathology of many women plays an important role in reproductive and gynecological inflammations [10]. Vaginitis is an opportunistic mucosal infection that affects three out of four women at least once during their reproductive years [11, 12]. It has been suggested that oxidative stress plays an important role in some physiological conditions and in many diseases, including vaginitis [13, 14]. Vaginal infection can be evaluated as a local mucosal infection similar to urinary tract infections, and likewise it is found to be effective on the oxidative stress. One study in rats had proved that vaginal candidiasis caused oxidative stress by damaging antioxidant enzymes, which revealed that the MDA plasma level decreased [15]. In diabetic patients with vaginitis, the down-regulated MDA level was also valid. Another study had shown that a main avenue of defense against fungal infection uses oxidative killing of microorganisms [16], but it remained unknown whether

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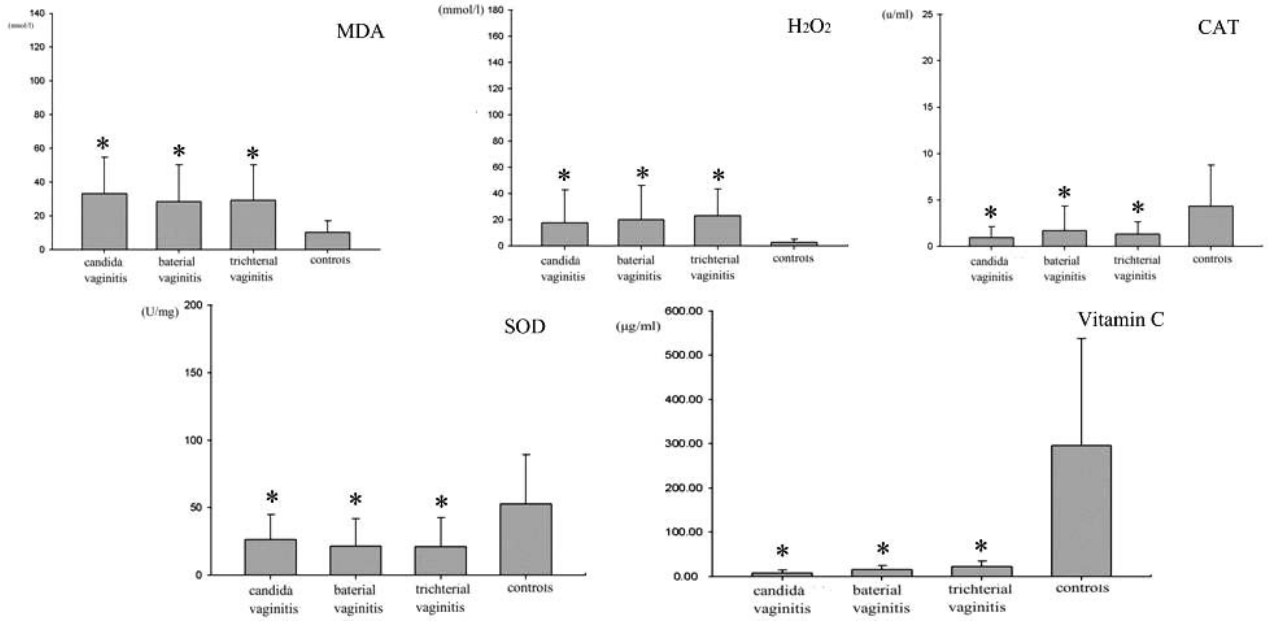

Figure 1. — The oxidative stress biomarkers in the several kinds of vaginitis before receiving therapy (*$p < 0.05$, compared to the healthy controls).

the oxidative stress was involved in bacterial vaginitis. In addition, there was no study on the level of oxidative stress in human vaginitis sample. Therefore, the aim of this study was to evaluate the effect of vaginal infection on the oxidative stress by using some oxidative biomarkers in the secretion of cervical samples, and elucidate the effect of oxidative stress on vaginitis in the vaginal microenvironment.

Materials and Methods

Patients

This was a prospective, clinical, and comparative study which was performed between April and August, 2010 in a single centre (Second Hospital of Lanzhou University, Lanzhou, GanSu, China) and involved in 257 patients who had gynecological evaluation within routine checkups or for vulvovaginal symptoms (increased vaginal discharge, genital itching, etc.).

The criterion of inclusion and exclusion were the following: all the subjects who were between 20 and 49 years of age and were in follicular phase. Individuals with pre-existing systemic diseases or chronic conditions, such as diabetes or an immunological disease (human immunodeficiency virus or systemic lupus erythematosus), were excluded. The subjects in pregnancy were also ruled out. All subjects enrolled in the study required regular menstrual cycles, refrained from douching, vaginal medications, and no sexual intercourse in last three days before the examination. Furthermore, all patients were refrained from systemic antimicrobial, antifungal drugs, and undergoing chemotherapy and immunosuppressive agent within the previous 30 days.

Based on diagnosis and microbiological examination [17] standardized by vulvovaginal symptoms, all the subjects were divided into four groups: 1) patients with candida vaginitis; 2) patients with bacterial vaginosis; 3) patients with trichomonas vaginitis; and 4) 60 healthy females were also involved in this study as the normal controls. The study protocol and informed consent document were reviewed and approved by the University of Lanzhou Institutional Review Board. Documented informed consent was obtained from all subjects prior to participation in this study.

All the patients received the following therapy against the different vaginitis, respectively: the patients with trichomonas vaginitis were treated with dispersible 500 mg oral ornidazole twice a day for seven days; the patients with candida vaginitis were treated with 0.15 g oral fluconazole twice a day, and miconazole nitrate vaginal suppositories (0.4 g) was inserted vaginally once a day for three days; the bacterial vaginosis patients vaginally received 0.2 g metronidazole vaginal effervescent tablets once a day for seven consecutive days. The therapeutic efficacy was evaluated at 21-35 days after the beginning of treatment, and all the patients had been cured after therapy.

Samples collection

A speculum was used to collect cervico-vaginal fluid from posterior fornix of the vaginal canal. All samples were collected and used the same test system which contained two swabs. Using these swabs, two vaginal samples were taken from each individual. One cotton swab was placed in a tube containing one ml of sterile saline and sent to be evaluated microscopically for the presence of candida sp., trichomoniasis, and "clue" cells. The other cotton swabs (pre-weighed) were placed in a tube containing two ml of sterile saline and stored at –70°C for further MDA, SOD, CAT, H_2O_2, and vitamin C detection.

Assay of oxidative stress biomarkers

About 100 ul of 8.1% sodium dodecyl sulfate (SDS) was added to dissolve the secretion in the swab, then vortexed, and incubated for 10 min at room temperature. Then 375 ul of 20% acetic acid (pH 3.5) and 375 ul of thiobarbituric acid (0.6%)

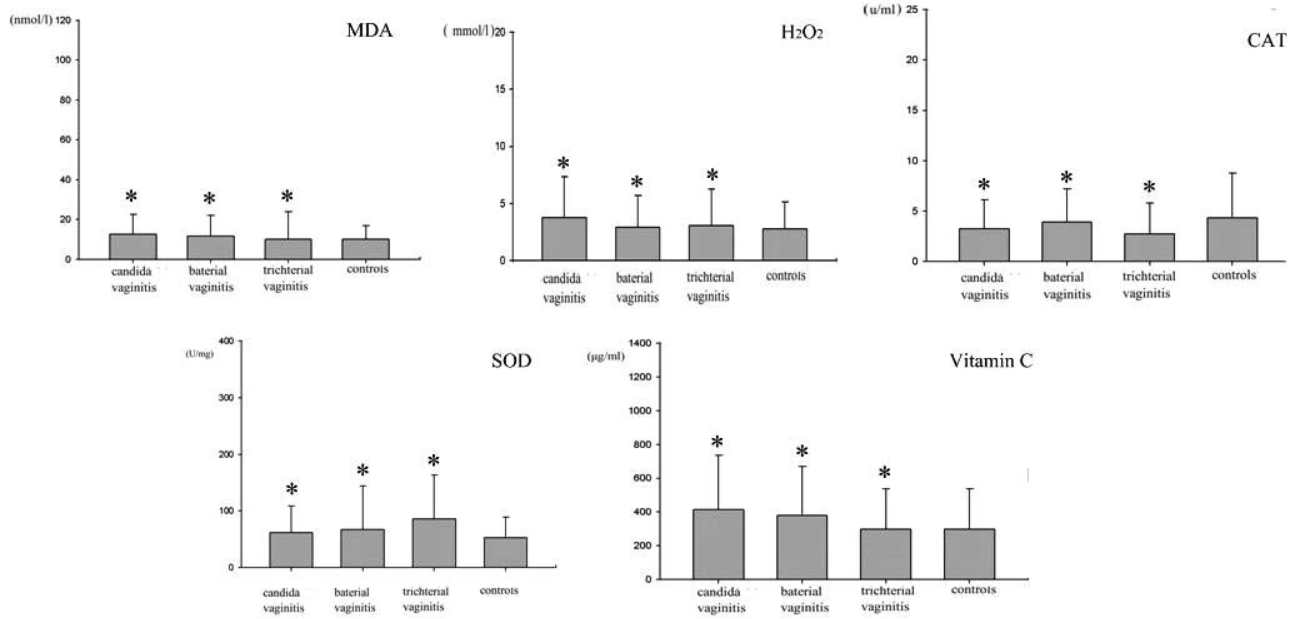

Figure 2. — The oxidative stress biomarkers in the several kinds of vaginitis after receiving therapy.

were added. Placing the sample in a boiling water bath for 60 min and cooled at room temperature. Finally 1.25 ml of butanol: pyridine (15:1) was added, and after vortexed and centrifuged at 1,000 rpm for five min, and 750 ul of the organic pink layer was measured at 532 nm. 1, 1, 3, 3-tetraethoxypropane was used as a standard.

Evaluation of SOD activity was determined. In brief, the assay mixture in a final volume of three ml contained sodium pyrophosphate buffer (0082 M, pH 8.3), phenazine methosulphate (186 mM), nitro blue tetrazolium (300 mM), nicotinamide adenine dinucleotide (NADH) (780 mM), diluted enzyme preparation, and distilled water. The reaction was initiated by the addition of NADH, following incubation at 37.8°C for 90 s. The reaction was stopped by adding one ml glacial acetic acid and the reaction mixture was vigorously shaken with four ml of n-butanol. The mixture was allowed to stand for 10 min, centrifuged, and butanol layer was separated. The colour intensity of the chromogen in butanol was measured at 560 nm against butanol over a spectrophotometer. A mixture without enzyme preparation was run in parallel to serve as control. The SOD activity was expressed in units/mg protein. One unit of the enzyme was the amount required to inhibit the rate of chromogen formation by 50%.

CAT activity was measured according to the Aebi method. Using a molar extinction coefficient of 43.6 M^{-1}/cm, the rate of the first 30 seconds was used to calculate the activity. Catalase activity was expressed as U mg^{-1}/Hb. The H_2O_2 concentration and the vitamin C level in the secretion of the vagina were both determined. To investigate the level of vitamin C, trichloroacetic acid was added to dissolve the secretion in the swab, then centrifuged at 3,000 g, for 10 min. Next, the mixture dissolved in 2-4 dinitrophenylhydrazine (DNPH) thiourea-copper sulfate, 37°C water-bath for four hours, following the addition of sulphuric acid (0°C), then let stand for 30 min at room temperature. The solution of optical density was measured at 560 nm to evaluate the level of vitamin C. To evaluate the H_2O_2 level, one ml PBS (0.01 M, pH 7.0) was added to dissolve the secretion in the swab, and 100 ul assay mixture was dissolved in the three ml solution containing xylenol (2.56 mM) and ammonium ferrous sulfate (250 mM) at a 9:1 dilution. The mixture was shaken for 5 s, let stand for 30 min at room temperature, then centrifuged at 2,000 g, for 10 min. Supernatant of optical density was measured at 560 nm to evaluate the level of H_2O_2.

Statistics

The descriptive statistics for each of the variables were calculated. Before analysis, each variable was examined for its distributional characteristics. All the data in the figures and tables are shown as means (± SD). Variation of the oxidative stress markers levels in the results obtained between two groups was calculated by Bonferroni test. The difference of the level of markers before treatment and post-treatment was calculated by paired t-test.

All the above hypothesis tests were two-sided, and a two-tailed p value of 0.05 or less was considered to be statistical significance.

Results

A total of 257 female patients (aged 20-49 years) with vaginitis were involved in this study. Meanwhile, a total of 60 healthy females aged 24-47 years served as the control group. Moreover, there was no significant difference in ages among the four groups ($p > 0.05$).

MDA

The results revealed that the MDA level in all the vaginitis patients including candida vaginitis, bacterial vaginosis, and trichomonas vaginitis was much higher than the normal controls, respectively (Table 1), while there was no significant difference in MDA level among

Table 1. — *Oxidative stress level in vaginitis and normal controls.*

	Vaginitis (n = 197)	Controls (n = 60)	*p* value
MDA	33.43 ± 21.50	10.18 ± 6.76	0.000
H_2O_2	20.05 ± 24.22	2.78 ± 2.34	0.000
CAT	1.31 ± 1.88	4.32 ± 4.46	0.000
SOD	23.18 ± 20.11	52.71 ± 36.61	0.000
Vitamin C	14.83 ± 11.32	297.57 ± 239.44	0.000

Table 2. — *The level of oxidative stress biomarkers pre- and post- treatment and statistical analysis.*

	Pre-treatment	Post-treatment	*p* value
MDA	25.70 ± 20.93	11.28 ± 10.43	0.000
H_2O_2	16.01 ± 22.45	3.15 ± 3.04	0.000
CAT	2.01 ± 2.99	3.57 ± 3.48	0.000
SOD	30.07 ± 27.85	66.60 ± 62.21	0.000
Vitamin C	80.84 ± 166.35	352.38 ± 280.73	0.000

the four groups of vaginitis before received treatment (Figures 1 and 2). MDA levels were much lower after therapy (Table 2).

H_2O_2

The results also showed that the H_2O_2 level of all the vaginitis increased before treatment compared with normal control (Figure 1). There was no significant difference among the candida vaginitis, bacterial vaginosis, and trichomonas vaginitis groups before treatment. Similarly, after receiving therapy, the H_2O_2 level decreased (Table 2), and there was no significant difference among the several kinds of vaginitis. Moreover, H_2O_2 pre-treatment level was much higher than the post-treatment level (16.01 ± 22.45 vs 3.15 ± 3.04, $p = 0.000$).

CAT, SOD, and vitamin C

CAT level, SOD, and vitamin C levels of the three groups of vaginitis decreased respectively in the pre-treatment vaginitis, and were all lower after the vaginitis was cured (Figures 1 and 2). Moreover, in contrast to the normal controls, there was no significant difference in H_2O_2, CAT, SOD, and vitamin C levels after receiving therapy, respectively (Table 1). All the levels of oxidative stress biomarkers pre- and post-treatment are also shown in Table 2.

Discussion

Free radical damage and oxidative stress are not "diseases". In fact, they are the by-product of normal cellular activity during the inflammation processes. Lipid peroxidation is one of the best parameters used for indicating the level of ROS-induced systemic inflammation damage. Persistent oxidative stress has a dramatic impact on immunological, clinical, and nutritional status [18, 19]. It has been previously shown that the pyelonephritis populations is oxidative stressed and to have significantly lower antioxidant concentrations than normal individuals [20].

In this study, the authors observed increased MDA and H_2O_2 levels in vaginitis compared to controls. This was associated with the corresponding decrease in CAT, SOD, and vitamin C levels. The authors therefore speculated that the oxidative stress would play an important role in vaginitis. These results are generally in agreement with some other studies. One study discovered that SOD activity could be a compensatory mechanism of cells that can suppress the superoxide radicals to combat the oxidative stress after Japanese encephalitis virus infection, and they concluded that SOD is involved in scavenging free radicals. Another study found that SOD and CAT together take part in stepwise oxygen reduction [21, 22]. In some other studies addressing liver injury [23], vivax malaria [24], peritonitis [25], the increased MDA level, and decreased SOD level were observed. Hence the increased MDA and H_2O_2 concentrations and the changes in the activities of the antioxidant SOD, vitamin C, and CAT showed the presence of oxidative stress in vaginitis, due to the infection, via an imbalance between antioxidants and pro-oxidants. In a state of oxidative stress, biological systems are not protected against the oxidative radical challenge that could result in toxic damage or death of the tissues and cells [26].

The elevated MDA and H_2O_2 concentrations in vaginitis in the present study could be attributed to increased ROS production, resulting in lipid peroxidation. The authors speculated that the infection would induce oxidative stress, such as excessive production free radicals, then destroy the mucosa, and lead to the necrosis of epithelium. Also, the excessive oxidative excess would influence neutrophil migration and healing of mucosa. The results obtained testify that the high levels of MDA and H_2O_2 in pre-treatment vaginitis may be the result of mucosa cell destruction by endotoxins. Actually, MDA level, a secondary product of lipid peroxidation, was frequently used as a biomarker of oxidative damage to lipids [13]. MDA and lipid peroxides are themselves free radicals with large reaction constants, which lead to oxidative damage, as modifications of proteins such as protein carbonylation. Thus it is possible that the increase in MDA level might have enhanced a system for the detoxification of lipid hydroperoxides [24, 25].

The authors found evidence of oxidative damage as indicated by increased H_2O_2 and decreased SOD and CAT in vaginitis. Hence, SOD is beneficial only in the presence of sufficient H_2O_2-detoxifying enzymes, such as catalase [27]. SOD and CAT are set to maintain the lowest possible levels of ROS in the cell, and is recognized as an essential component of an organism's self-maintenance [28]. Thus Sheng R. *et al.* reported a concomitant increase in lipid peroxidation and a drop in antioxidant enzyme activities of SOD and CAT in the rat's cardiomyocyte [29]. These data demonstrate that enhanced activities of SOD, CAT, and vitamin C can lead to the elimination of ROS. In particular, as other authors have shown, SOD may eliminate organic hydroperoxide from cells and defend cells from potential damage from the products of lipid peroxidation. In the

present study, the decrease in SOD, CAT, and vitamin C activities in vaginitis was observed simultaneously with the increased concentration of MDA. The lower SOD, CAT, and vitamin C activities were probably due to enhanced ROS production [30]. This finding may prove that anti-oxidant material is involved in the inactivation of toxic lipid peroxidation products accumulated during destructive processes in the early stage of infection. The authors assumed that the increased activity of SOD would result in an increased H_2O_2 concentration and consequently in a further increase in CAT activity. CAT is known to be inhibited by the accumulation of superoxide anion during destruction processes in the gut [31]. When vaginitis was cured, there was no statistical difference in the levels of MDA, H_2O_2, SOD, CAT, and vitamin C between the patients and healthy controls, which supports the study's hypothesis. The authors speculated that with the inflammation that vanished, necrosis of mucosa cells and macrophages would not generate; then the level of oxidative stress and antioxidase decreased accordingly. Oxidative stress, resulting either from increased oxidant production and reduced antioxidant levels, appears to be a basic mechanism in vaginitis processes and progressive inflammation in patients [32]. The present study indicated that pathogens induce vaginitis and cause oxidative damage.

Vaginitis treatment has been difficult, because its etiology, pathology, microbiology, and transmissions are not clear; therefore, its pathogenesis is particularly important. The data demonstrated that the oxidative stress was involved in the inflammation response of vaginitis. Some studies found that trichomoniasis can up-regulate the expression of nitric oxide (NO) and inducible nitric oxide synthose (iNOS) expression in monocyte macrophages [33, 34], which were both the pivotal molecule of the oxidative stress. One random clinical trial completed by Khajehei [35] confirmed that vitamin C supplement would enhance the curative effect of vaginitis by inhabitation of the oxidative stress, so the anti-oxidation therapy would benefit vaginitis. Besides, leucorrhea microscopy is always the important method for the diagnosis of vaginitis, but the diagnostic method had a low specificity and sensitivity, impacted by the smear results. However, the oxidative stress biomarkers including MDA, CAT, SOD, etc. were the potential markers for the diagnosis of vaginitis. The authors have found the biomarkers varied significantly with vaginitis, compared to the controls, and with the progression of treatment, these biomarkers changed accordingly. Then the markers all were quantitative, and changed with the medical treatment. So compared to leucorrhea microscopy, the oxidative stress biomarkers may be a more effective method for vaginitis diagnosis, and could monitor the treatment effectiveness more accurately.

There were several limitations in this study. One of them is that the influence of drug on the oxidative stress could not be excluded in vaginitis. In addition, the effect of the oxidative stress needs further study. This study demonstrated the oxidative stress biomarkers were involved in the infection process, but the effect of free radicals on the epithelial cells or the pathogen was still unclear.

In conclusion, the changes in MDA and H_2O_2 levels as well as the altered activities of the antioxidant enzymes SOD and CAT and vitamin C in vaginitis may be useful evidence for impaired antioxidant status and the occurrence of oxidative stress in vaginitis.

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