Oxidative Stress Biomarkers in Cervical Mucus: A Potential Diagnostic Indicator for Pathological Conditions

Rina Adisty, Muhammad Alwi, Wahidah Nur Hasanah, Abdul Rohman Hakim
Department of Pathology and Toxicology, Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia

ABSTRACT
The objectives of this study were to measure several oxidative stress parameters in cervical mucous with several pathological conditions. The parameters are myeloperoxidase (MPO) activity, hydrogen peroxide (H_2O_2) and Advanced Oxidation Protein Products (AOPPs) levels. Seventy-six women with aged 20-45 years, who attend to Indonesian Cancer Association, Jakarta, Indonesia were enrolled in the study. Cervical mucus was taken from each patient, and after that, pap smear procedure is performed to determine the condition of each patient's cervix. According to the results of the pap smear, there are three cervical conditions, such as normal, mild chronic specific infection, chronic non-specific infection, and atypical cells of undetermined significance (ASCUS). A significant increase in MPO activity, H_2O_2, and AOPPs levels were observed in all group of patients, compared to normal patients. In conclusion, oxidative stress was involved in several pathological conditions in the uterine cervix, and the parameters measured in this study may be used as a marker in those conditions.

Keywords: Cervix, Cervical Mucus, Oxidative Stress.

INTRODUCTION

The cervix located in the lower part of the uterus. The shape is cylindric, projects through the superior-anterior vaginal wall, and connected with vagina via canalis endocervical, which is located at the top of vagina[1]. The cervix has several important functions such as, prevent the pathogens from the vagina into a uterus, allows the sperm to move into the fallopian tubes, and maintaining a pregnancy until the childbirth. It is weel known that in order to carry out some of these functions, the cervix produces cervical mucous[2].

Human cervical mucous produced by cervical crypts. Mostly aqueous, because it content 90-95% water, and increases during the periovulatory period until 98-99%. This mucus is composed of two parts, including a part of a more dilute containing inorganic ions, proteins, enzymes, immunoglobulins, amino acids and sugars, and more viscous part containing high-molecular-mass components[3]. It is well known that the condition of the cervical mucus was greatly influenced by a number of circumstances, such as hormonal state and the presence of several pathological conditions in the feminal genital tract[4]. This makes this mucous can be used as a marker for several pathological conditions, with the low cost and the easier method for the sample collection[5].

**Author for Correspondence: esuhartono@unlam.ac.id*

Oxidative stress is defined as the unbalancing between the production of free radicals and active intermediates, and the antioxidant which is known as a system to neutralize and eliminate them[6]. Oxidative stress has been linked to several pathological conditions such as atherosclerosis, hypertension, ischemia/perfusion, diabetes, stroke, Parkinson's disease, rheumatoid arthritis, cancer, Alzheimer disease, etc[7-8]. Oxidative stress also linked with several feminal genital tract pathological conditions, including endometriosis, woman infertility, polycystic ovary syndrome, spontaneous abortion, preeclampsia and eclampsia, etc.[9]. Measurement of oxidative stress parameters may be useful to assess other pathological conditions in the female genital tract, such as abnormalities in the cervix.

In this study, we tried to assess multiple parameters of oxidative stress in the cervical mucous such as the level of hydrogen peroxide (H_2O_2), and advanced oxidation protein products (AOPPs), and the activity of myeloperoxidase (MPO). The results are expected to be used as supplementary information how the oxidative stress pathomechanism is involved in several pathological conditions in the uterine cervix. Also, the results of this study are expected to serve as information to define a new marker for several pathological conditions in human cervix.

MATERIAL AND METHODS

Patients and Sampling

This study was performed at the Anatomy Pathology Laboratorium of Ulin General Hospital, and Medical Chemical/Biochemical Laboratorium of Lambung Mangkurat University, South Kalimantan, Indonesia. Before this study, the approval of the Ethical Committee of Lambung Mangkurat University, Banjarmasin, South Kalimantan, Indonesia was obtained. A total of 76 women aged 20-45 years, who attend to Indonesian Cancer Association, Jakarta, Indonesia were included in the study. Written informed consent was obtained from each patient before the study was started.

Before sample collection, speculum was inserted into the vaginal cavity to expose the cervix uteri before any other vaginal examination. The cervical mucous form each patient was collected using a cervical brush. A conventional pap smear was made first, then the remainder of the cellular material on the cervical brush was rinsed into a vial containing PreservCyt preservative fluid (Cytyc Corporation, Boxborough, MA, USA). The conventional Pap smear was forwarded to the Anatomy Pathology

Laboratorium of Ulin General Hospital, while the vial was forwarded to the Medical Chemical/Biochemical Laboratorium of Lambung Mangkurat University for the measurement of oxidative stress parameters such as the activity of MPO and the level of radical superoxide, H_2O_2, and AOPPs. *MPO Activity Analysis*

The activity of MPO was analyzed using the spectrophotometrically method, as described previously. The analysis is based on the oxidation of o-dianisidine in the presence of H2O2 as an oxidizing agent, which was catalyzed by MPO. The oxidation reaction will produce a brown color product, that can be measured spectrophotometrically at 470 nm. The MPO activity was expressed as 1 µmol of H2O2 that is degraded per minute at 25oC[10]. *H_2O_2 Concentration Analysis*

The H_2O_2 concentration analysis was calculated by the ferrous oxidation in xylenol orange (FOX) methods with slight modifications. For measuring the H_2O_2 level, we used three different mixture, which is assay, standard, and blank mixture. The assay mixture contained 200 µL of sample, 160 µL PBS pH 7.4, 160 µL $FeCl_3$ (251.5 mg $FeCl_3$ dissolved in 250 ml distilled water) and 160 µL ophenanthroline (120 mg o-phenanthroline dissolved in 100 ml distilled water), while the standard mixture contained all the same chemicals with the assay mixture, except for the sample is replaced by 200 µL of 1 M H_2O_2. The blank mixture contained all the same chemicals with the assay mixture, except for the absence of $FeCl_3$. Then, all the mixture were incubated for 30 minutes at room temperature and then centrifuged at 12,000 rpm for 10 minutes. Furthermore, measured the absorbance of each mixture with the spectrophotometer at a wavelength 505 nm. H_2O_2 concentrations were expressed as mM[10-11].

AOPPs Concentration Analysis

AOPPs concentration analysis was calculated by spectrophotometric methods. 200 µl of a sample were diluted with phosphate buffer solution. Then, placed on 96test wells. Add 20 ml of acetic acid in each test well. For the standard, add 10 ml of 1.16 mol potassium iodide, 200 ml of chloramine-T solution (0–100 mmol/l), and 20 ml of acetic acid. Placed the standard mixture into standard wells. Then, read the absorbance of the mixture at 340 nm. The absorbance was read against a blank solution. A blank solution is a mixture between 200 ml of phosphate buffer solution, 10 ml of potassium iodide, and 20 ml of acetic acid. AOPP concentrations were expressed as mmol/l of chloramine-T equivalents[11-12].

Statistical Analysis

The data were presented as the mean – standard deviation. Statistical analyses were carried out using the Microsoft excel 2010 and SPSS version 16 software package for windows 10. The one-way ANOVA test and followed by Pot Hoc Tukey HSD test with 5% of significance was used to compare the mean of measured parameters values between the studied patient groups.

RESULTS

Before the oxidative stress parameters measurement in cervical mucus, pap smear procedure is done to assess the cervix condition of each patient. From the pap smear, patients were classified into 4 groups, among others normal, mild chronic specific infection, chronic nonspecific infection, and atypical cells of undetermined significance (ASCUS) (figure 1a-d). The number and percentage of patients from each group are presented in Table 1.

In this study, the first oxidative stress parameters that investigated is MPO activity. A significant increase in MPO activity in cervical mucus was observed in all group of patients, compared to the normal patients (figure 2). The level of H_2O_2 and AOPPs level were determined. The results show in figure 3, and 4, respectively. A significant increase in H_2O_2 and AOPPs levels were observed in all group of patients, compared to normal patients.

DISCUSSION

Recently, biological fluids have been used as sources to define markers for several pathological conditions include cervical mucus. Mania-Pramanik et al.[13] use lactoferrin as a marker in cervical mucus for reproductive tract infection and intrauterine device. Szczepanska et al.[14] results study suggest that interleukin-6 (IL-6) in cervical mucus can use as a sensitive marker of viral infection in women with a fetal defect. Holst[15] propose a cervical mucus to predict the cases with high risk of Pre-term birth and infection that require action from those at low risk that should not be hospitalized and exposed to the unnecessary intervention. In this present study, we propose oxidative parameters to evaluate several pathological conditions in the cervix. Several oxidative parameters were measured in this study including MPO, H_2O_2, and AOPPs. The results show that all parameters were increased in all pathological conditions that investigated in this study. There is three pathological conditions, among others mild chronic

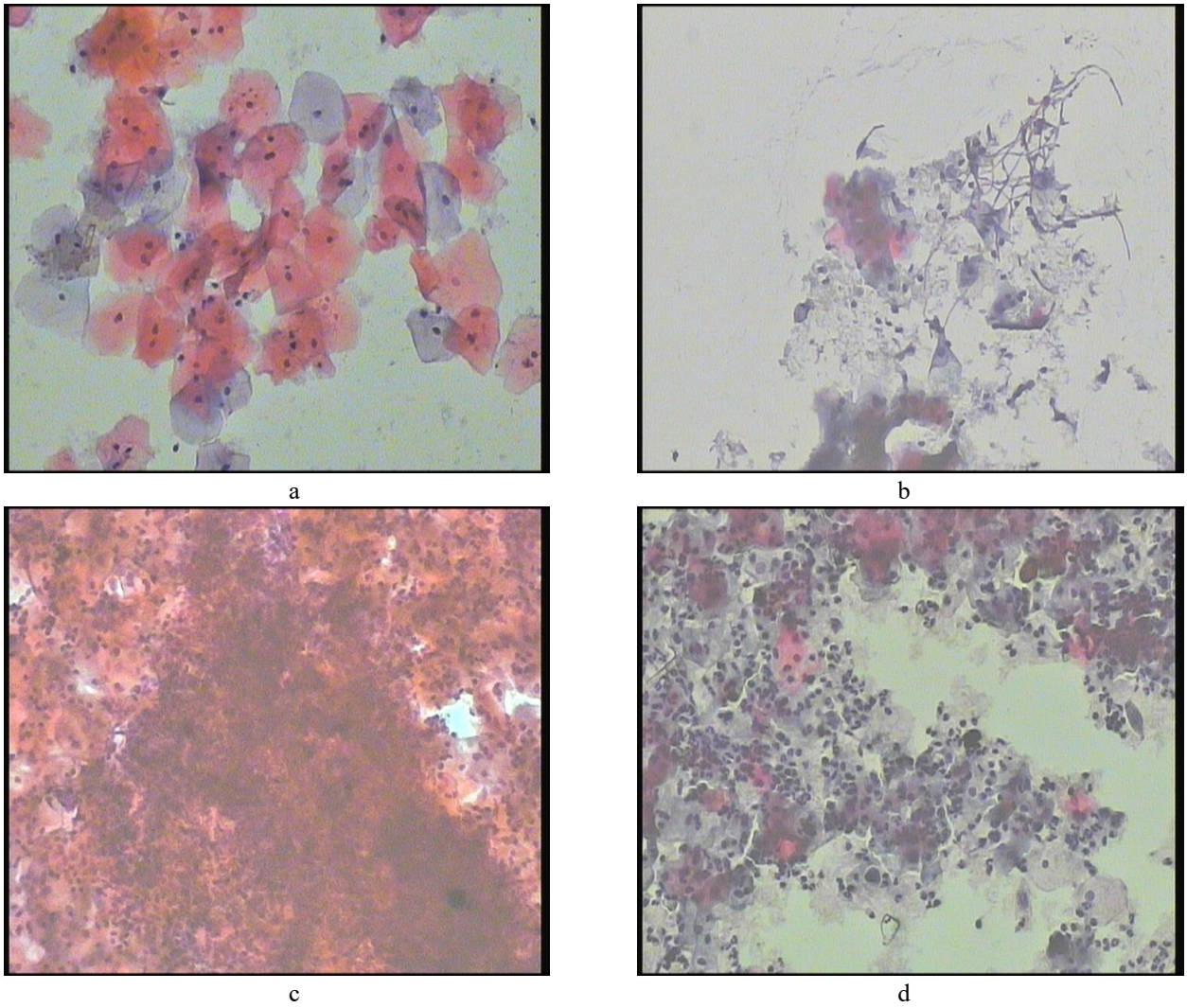

Figure 1: Pap smear test results; (a) normal pap smear; (b) mild chronic spesific infection; (c) chronic non-spesific infection; and (d) Atypical Cells of Undetermined Significance (ASCUS).

Table 1: The number and percentage of patients based on the results of pap smear.

Groups	Normal	Mild Chronic Spesific Infection	Chronic Non-Spesific Infection	ASCUS
Number of patients	52	4	9	3
Percentage	76%	6%	13%	4%

ASCUS: Atypical Cells of Undetermined Significance

specific cervix infection, chronic specific cervix infections, and ASCUS.

The results suggest that oxidative stress involved in cervical infection and cell changes. This proves that an infection both specific or non-specific, and changes in cervical cells causes molecular changes in cervical mucus. For infection, it is long known that MPO is involved in bacterial killing and oxidative injury in the host. This is known as MPO-hydrogen peroxide chloride H_2O_2-Cl system, that is important to antimicrobial system[16]. For ASCUS condition, as far as we know, there have been no investigations of the association between MPO activity and ASCUS condition. However, there are several studies linking the activity or levels of MPO with Human Papilloma Virus (HPV) infection in the cervix and cervical cancer lesions[17-18].

The results of this present study also indicated that several pathological conditions in cervix induced the formation of ROS in cervical mucus. It can be seen from the increasing of H_2O_2 level in cervical mucus. The increasing of ROS in cervical mucus might be due to rapid respiratory burst mechanism of phagocytic cells by NADPH oxidase[19]. Since the samples taken from the cervical mucus in chronic specific and not-specific infections condition, the respiratory burst mechanism may play a role in the increasing of ROS production. Also, since the ASCUS might be related to HPV infection, this mechanism as mentioned earlier may play a role too in the increasing of ROS production in ASCUS condition. NADPH oxidase activation in phagocytic cells can lead to the reduction of oxygen to form radical superoxide. Radical superoxide

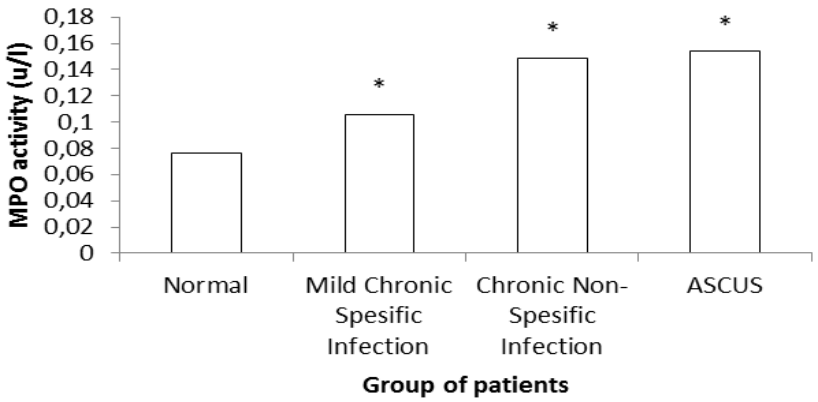

Figure 2: Comparison of MPO activity between group of patients. * Values differs significantly from normal patients (P<0.05). ASCUS: Atypical Cells of Undetermined Significance.

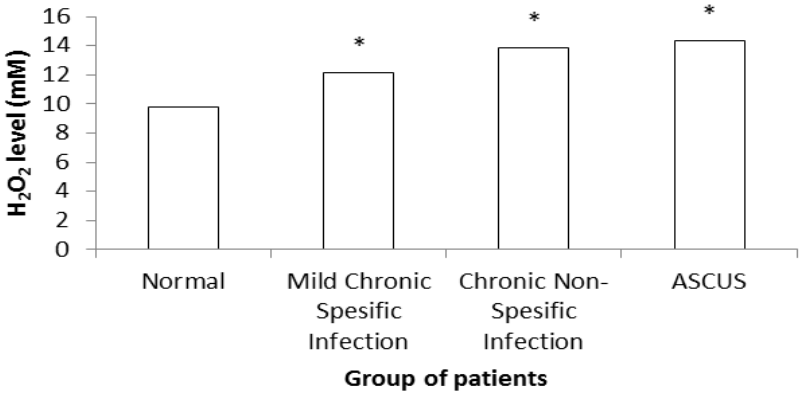

Figure 3: Comparison of H_2O_2 level between group of patients. * Values differs significantly from normal patients (P<0.05). ASCUS: Atypical Cells of Undetermined Significance.

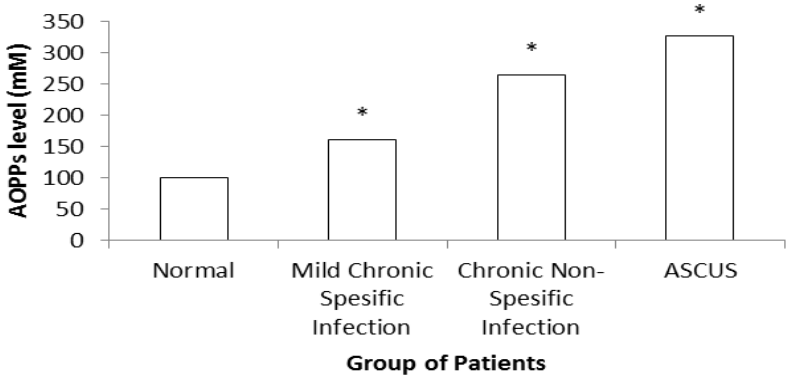

Figure 4: Comparison of AOPPs level between group of patients. * Values differs significantly from normal patients (P<0.05). ASCUS: Atypical Cells of Undetermined Significance.

then dismutated by superoxide dismutase (SOD) to form H_2O_2, hydroxyl anions, and hydroxyl radicals via the Fenton and Haber-Weiss reaction[10,20]. It is well documented that the oxidative stress state is associated with damage to all biomolecules, including protein. One of the possible marker for protein damage due to oxidative stress is AOPPs[21]. It was first described by Witko-Sarsat et al.[22]. who found the increasing of protein damage in the plasma of uremic patients. The result of this present study suggests that AOPPs level was increased in the cervical mucus of several pathological conditions in the cervix that investigated in this study. The increasing of AOPPs level might be due to the increasing level of ROS and the activity of MPO. The ROS can react directly with proteins or via activation of MPO which will produce chlorinated oxidants. Both direct reaction or chlorinated oxidants could react with protein which resulted in the formation of AOPPs[23].

In conclusion, these result suggested that oxidative stress was involved in several pathological conditions in the uterine cervix, and the parameters measured in this study may be used as a marker in those conditions. Further investigations might be needed to elucidate the exact mechanism how those conditions could induce oxidative stress in cervical mucus.

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