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# Evaluation of dynamic thiol–disulfide balance in preinvasive lesions of the cervix

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## Abstract

**Purpose** This study aimed to determine the potential clinical use of dynamic thiol–disulfide balance in cases with preinvasive lesions of the cervix.

**Methods** One hundred and sixteen patients with high-grade squamous intraepithelial lesion, 100 patients with low-grade squamous intraepithelial lesion, and 110 healthy controls were enrolled in the study. A fully automated colorimetric system was used to determine the levels of thiol–disulfide parameters. The ischemia-modified albumin, total oxidant–antioxidant capacity, and oxidative stress index of the retrieved cases were further analyzed.

**Results** Native thiol and total thiol levels are significantly lower in the high-grade squamous intraepithelial lesion group according to control group ($p$: 0.004 and 0.015, respectively). Disulfide level is significantly increased in the high-grade squamous intraepithelial lesion group compared to control group ($p$: 0.004). Oxidative stress index levels in high-grade squamous intraepithelial lesion group were observed as significantly higher according to the control group ($p$: 0.014). Ischemia-modified albumin levels in the high-grade squamous intraepithelial lesion group were observed as significantly higher compared to the control group ($p$: 0.020). Disulfide levels are positively correlated with risk type of Human papillomavirus ($r$: 0.420, $p < 0.001$).

**Conclusion** The analysis of dynamic thiol–disulfide balance revealed considerable oxidative damage in patients with Human papillomavirus-related cervical precursor lesions compared to women with ordinary cytology specimens. Therefore, investigation of thiol–disulfide balance with presented method represents a new promising test for early diagnosis and management of women at high risk for cervical cancer.

**Keywords** Cervical preinvasive lesion · Disulfide · Oxidative stress · Squamous intraepithelial lesion · Thiol · Disulfide homeostasis

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## Introduction

Precancerous cervical lesions associated with Human papillomavirus (HPV) are known to be the most important causes of cervical cancer development. The risk of developing cervical intraepithelial neoplasia 3 + after treatment or after initial negative surveillance is not low, especially in the group of patients with precancerous lesions detected in the 30–64 age range [1]. For this reason, diagnosis of precancerous lesions and monitoring of possible progression are the main strategies for prevention of invasive cancer development. According to the 2012 Lower Anogenital Squamous Terminology working group consensus, they agreed to classify cervical precancerous lesions as 2 stages (low-grade squamous intraepithelial lesion (LSIL)/high-grade squamous intraepithelial lesion (HSIL)) [2]. There is not any biomarker that will be a definitive diagnostic tool for cervical cancer or for leading lesions, recently. Therefore, biomarkers that could predict cervical precursor lesions or guide progression of these lesions are highly desired.

Reactive oxygen species (ROS) that occur during various intracellular metabolic events cause oxidation effect in cells. Therefore, ROS are important source of oxidative damage that cells are exposed to [3]. ROS or free radicals released during intracellular events might be hazardous to DNA structure. Thus, the resulting DNA damage can initiate the process of carcinogenesis [4]. In addition, free radicals with this oxidized property can disrupt oxidation–reduction events that occurs in cells physiologically. As a result, the oxidation–reduction balance is impaired. SH (thiol): S–S (disulfide) balance in the body is an important redox event that reflects the oxidation–reduction balance. It has been demonstrated by various studies that imbalance of this redox state causes many diseases, including cancer [5]. Moreover, ROS in cervical tissue are known to cause tissue damage [6].

In this study, plasma thiol/disulfide homeostasis was evaluated using the process of Erel et al. described previously [7]. With this method, the levels of free thiol, total thiol and disulfide in plasma can be accurately calculated in as little as 10 min without any need for separation. This is a completely automated, easy and inexpensive method. In addition, a growing amount of literature suggests that an abnormal TD balance may be a factor underlying the pathogenesis of diverse malignant states including endometrial, cervical, colon, lung and breast cancers [8–12]. Thus, determination of dynamic TD homeostasis can offer precious information on certain precancerous states of malignancies.

The role of oxidative stress were assessed in some previous investigations in patients with precancerous cervical lesions, but TD hemodynamics have not previously been examined in these patients [13]. Therefore, the objective of the current study was to examine the status of plasma TD balance in patients with precancerous cervical lesions.

## Materials and methods

### Research design and subjects

This prospective cross-sectional study was organized and carried out at the Obstetrics and Gynaecology Department of Mugla Sitki Kocman University and Private Adatıp Sakarya Hospital. All the patients conducted in this study are referred by primary care units of public healthcare system or directly applied to our outpatient clinic. All laboratory works were carried out in the laboratory of Clinical Biochemistry Department of Yildirim Beyazit University, Ankara, Turkey. The diagnosis of cervical preinvasive lesions was conducted using Bethesda system by the pathology experts. All patients being asked to be involved in the research if they already applied with Papanicolaou test between September 2018 and July 2020. The ones who accepted to be involved in all steps of this study and meeting the age criteria of being 21 years or over are met the inclusion criteria. The HSIL, LSIL patients and healthy controls were excluded if they had cognitive impairment, diagnosed malignancy, major medical or surgical illness, dietary antioxidant intake, alcohol and smoking use, and drug addiction. One hundred women formed the group with LSIL. One hundred and sixteen women formed the group with HSIL. Among whom cytopathological exam test result were normal who are age-matched 110 women are accepted as the control group. Serum TD and other oxidative stress parameters were determined in all volunteers.

### Epidemiological data

Epidemiological data were obtained from the hospital database of patients such as age, body mass index, gravidity, parity, and HPV status. Missing data were derived on the day of the gynecological exam directly from the patients. The patients were then categorized as HPV negative, low-risk HPV or high-risk HPV.

### Human papillomavirus detection and typing

We used a commercial kit; digene HC2 HPV DNA Test (Qiagen Germantown, Inc., MD, USA) for HPV typing. This kit can detect 13 types of high-risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and 5 types of low-risk HPVs (6, 11, 42, 43, and 44).

### Blood collection and sample preparation

For laboratory analysis, 6-ml preprandial blood samples were retrieved from the vein of the forearm of all participants at the outpatient clinic. After centrifugation of the blood samples, supernatant was transferred to a 1.5-ml Eppendorf tube and then preserved at − 80 °C until biochemical analysis.

### Biochemical data

The total thiol, native thiol and disulfide levels in the serum were calculated using a recent method developed by the authors Erel and Neselioglu [7]. To reach the conclusion of native thiol, total thiol and disulfide levels, we used spectrophotometry at 600 nm and *Rel Assay* Diagnostics© kit (Gaziantep, Turkey). The results are shown in µmol/L.

To reach the conclusion of TAC, TOC and OSI, we used spectrophotometry at 600 nm and *Rel Assay* Diagnostics© commercial kit. The units are shown in mmol trolox equivalent/L, mmol H2O2 equivalent/L and mmol trolox/L, respectively.

To reach the conclusion of IMA, we used the rapid and colorimetric method defined by Bar-Or et al. [14]. The results were expressed as IU/mL.

### Data analysis

Statistical analyses were computed with SPSS version 22 for Windows (SPSS Inc., USA). The normal distribution of the data was determined by the Shapiro–Wilk test. One-way analyses of variance (ANOVA) were employed to analyze data between three independent groups followed by least squares derivation post-hoc comparisons. The mean, standard deviation and percentages were given as descriptive statistics. Pearson correlation coefficient was used to determine the strength of the correlation between serum disulfide and HPV types. The significance was set at $p \leq 0.05$.

## Results

The demographic data of the study participants are shown in Table 1. There were no statistical differences in terms of age, BMI, gravidity and parity between the groups of control, LSIL and HSIL ($p$: 0.750, 0.128, 0.068, and 0.435, respectively).

In the control group, 5.45% of the patients were low-risk and 18.18% of the patients were high-risk HPV positive. Of the 100 LSIL patients, 22% were low-risk and 75% were high-risk HPV positive. Of the 116 HSIL patients, 11.20% were low-risk and 81.03% were high-risk HPV positive.

The outcomes of biochemical parameters in the three groups are compared and shown in Table 2. We observed that disulfide level is significantly increased in the HSIL group compared to the control group ($p$: 0.004). Native thiol and total thiol levels are significantly lower in the HSIL group compared to the control group ($p$: 0.004 and 0.015, respectively).

There were no statistical differences between the three groups in terms of TAC and TOC levels. But OSI levels in the HSIL group were observed as significantly higher according to the control group ($p$: 0.014).

Mean serum albumin levels were found lowest in the HSIL group and highest in the control group ($p$: 0.028). IMA levels in the HSIL group were observed as significantly higher compared to the control group ($p$: 0.020).

We found significant correlation between risk types of HPV and disulfide levels ($r = 0.420$, $p$: 0.001). According to this correlation, a significant increase in serum disulfide level was observed as the HPV condition progressed from negative to high risk (Fig. 1).

**Table 1** Comparison of demographical and clinical data among control, LSIL and HSIL groups

| Variables | | Control (mean ± SD) | LSIL (mean ± SD) | HSIL (mean ± SD) | $p$ value |
|---|---|---|---|---|---|
| Age (years) | | 43.02 ± 9.32 | 43.89 ± 8.32 | 43.36 ± 7.38 | 0.750$^{\Delta}$ |
| BMI (kg/m$^2$) | | 27.71 ± 2.03 | 27.42 ± 2.26 | 28.03 ± 2.36 | 0.128$^{\Delta}$ |
| Gravidity ($n$) | | 2.58 ± 1.30 | 2.25 ± 0,86 | 2.36 ± 0,95 | 0.068$^{\Delta}$ |
| Parity ($n$) | | 1.84 ± 1.15 | 1.81 ± 0.71 | 1.96 ± 0.78 | 0.435$^{\Delta}$ |
| HPV type (%) | Negative | 76.36 | 3 | 7.75 | |
| | Low-risk HPV | 5.45 | 22 | 11.20 | |
| | High-risk HPV | 18.18 | 75 | 81.03 | |

*LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, *SD* standard deviation, *BMI* body mass index, *HPV* Human papillomavirus

*Significant

$^{\Delta}$Not significant at 0.05 level; one-way ANOVA test

**Table 2** Comparison of biochemical measurements among preinvasive cervical lesions and control group

| Variables | Control (*n*: 110) Mean ± SD | LSIL (*n*: 100) Mean ± SD | HSIL (*n*: 116) Mean ± SD | *p* value |
|---|---|---|---|---|
| Disulfide (µmol/L) | 21.17 ± 4.78 | 21.71 ± 4.63 | 23.14 ± 4.50 | **0.004*** |
| Native thiol (µmol/L) | 312.55 ± 77.85 | 289.41 ± 49.58 | 279.03 ± 75.96 | **0.004*** |
| Total thiol (µmol/L) | 354.89 ± 80.88 | 332.84 ± 52.55 | 325.31 ± 75.44 | **0.015*** |
| IMA, (IU/mL) | 0.63 ± 0.18 | 0.64 ± 0.16 | 0.70 ± 0.19 | **0.020*** |
| Albumin(mg/dL) | 3.78 ± 0.32 | 3.72 ± 0.27 | 3.66 ± 0.42 | **0.028*** |
| TAC (mmol trolox equivalent/L) | 1.57 ± 0.23 | 1.55 ± 0.18 | 1.51 ± 0.22 | 0.099 |
| TOC (mmol $H_2O_2$ equivalent/L) | 15.12 ± 5.14 | 16.27 ± 4.42 | 16.47 ± 4.65 | 0.085 |
| OSI (mmol trolox/L) | 9.80 ± 3.53 | 10.68 ± 3.16 | 11.16 ± 3.16 | **0.014*** |

*LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, *SD* standard deviation, *TAC* total antioxidant capacity, *TOC* total oxidant capacity, *IMA* ischemia modified albumin, *OSI* oxidative stress index

*Significant difference between control and HSIL groups; significant at 0.05 level; one-way ANOVA test

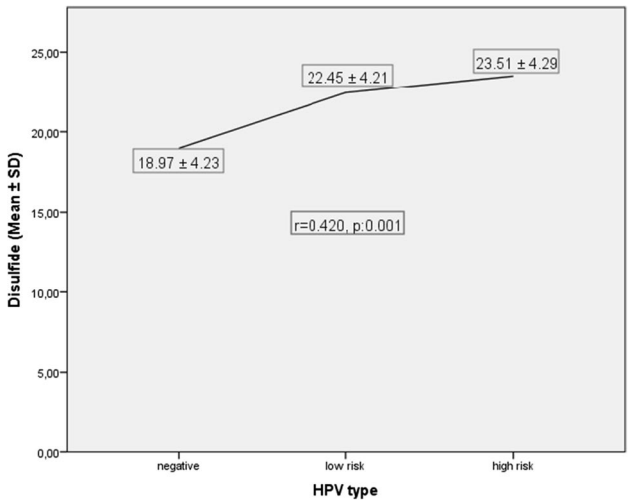


**Fig. 1** The serum level of disulfide showed a consistent increase with HPV type

## Discussion

HPV infection's contribution to the development of precancerous cervical lesions is known. Cervical cancer develops as a result of malignant transformation of preinvasive lesions. Only a small percentage of cases with HPV infection develop cervical cancer. It is thought that other factors beside infectious reasons may play a role in the pathophysiology of cervical cancer. In this context, the development of new biomarkers that can detect precursor lesions of the cervix will help elucidate the pathophysiology of these lesions. In our study, we investigated the balance of TD, which is indicative of oxidative stress, in precursor lesions of the cervix. According to our literature survey, we have never encountered such a study in this group before. Therefore, this research is the initial investigation of serum TD balance in patients with cervical precursor lesions. The main results of our study can be summarized as follows: cervical squamous intraepithelial lesions showed high levels of disulfide and low levels of native thiol and total thiol compared to the control group. Secondary results of our study showed an increase in IMA and OSI levels in patients with both LSIL and HSIL compared to the control group.

The role of oxidative stress in the formation of cervical preinvasive lesions has been proven, and there are several studies in the literature about it. Gonçalves and colleagues investigated serum thiol content levels in their study, which included 11 LSIL and 17 HSIL patients [15]. They found no difference in plasma thiol levels among patient groups. In this result, both the low number of patients in the study and the different thiol measurement technique may have been the probable causes. Cysteine protein is one of the low molecular weight elements of the plasma thiol pool. In their study, Goodman and colleagues found a weak link between plasma cysteine deficiency and LSIL risk, but found no association related to HSIL [16]. Glutathione is also a low molecular weight element of plasma thiol pool and several studies have also been conducted in which total glutathione and oxidized glutathione levels have been measured. In these studies, plasma total glutathione levels were reported as low and oxidized glutathione levels were reported as high in patients with preinvasive lesions compared to patients without preinvasive lesions [17, 18]. They added that this relationship may be related to HPV positivity. Slater and colleagues investigated the levels of the protein thiol in cervical tissue and reported that the ratio of the protein thiol epithelial/

stroma decreased in cervical dysplasia compared to normal cervical tissue [6]. This study indicated that the thiol groups of proteins are mostly concentrated in the epithelium layer of the cervix, and that the balance of the thiol groups between the epithelium and stroma may be a clue in the development of cervical dysplasia. In addition, Nöhammer and colleagues reported that the rate of thiol/disulfide decreased in the dysplastic cervix compared to the normal cervix in the study in which both thiol and disulfide levels were evaluated in cervical tissue [19]. As a result, they emphasized that thiol/disulfide balance in cervical tissue indicates impaired biochemical events occurring in dysplasia. In our study, we also found low serum total and native thiol levels and high disulfide levels in the LSIL and HSIL group compared to the control group, similar to those in cervical tissue studies. However, it is clear from the results given in Table 2 that there is a gradation of change in the serum levels found for total thiol, native thiol and disulfide in moving from normal through LSIL to HSIL. Our results demonstrate that deterioration in dynamic TD balance towards right shift is associated with the development and progression of cervical precursor lesions.

Cruz-Gregorio and colleagues found that E6 oncoprotein of HPV 16 and 18 reduced the levels of glutathione [20]. Reduced thiol content, such as glutathione, also prevents free radicals from being removed from the environment [21]. We also found that total thiol and native thiol levels decreased in the dysplastic group in parallel with these data. Decreased thiol levels confirm that cells' antioxidant defense mechanisms are weakened in HPV-associated preinvasive lesions.

ROS that are the source of oxidative stress have a stimulating role for mutation formation and neoplastic progression in HPV-infected cells [22]. In addition, HPV increases the ability of infected cells to live in an oxidative environment [23]. In some of the studies conducted on cervical preinvasive lesions in the literature, oxidative stress indicators were associated with HPV, while the relationship between preinvasive lesions were not determined [18]. In addition, there are investigations showing that oxidative stress is associated with both HPV and dysplasia grade [24]. In this study, we evaluated dynamic TD balance and found an association between both HPV and preinvasive lesions. However, there is a gradation of change in the serum levels found for disulfide in moving from normal through low-risk to high-risk HPV type. As a result, we found a moderate and positive correlation between disulfide and HPV type in our correlation analysis. In particular, participants with high-risk HPV infections revealed higher levels of disulfide levels than participants with low-risk HPV infections. This outcome highlights the role of increased oxidative stress in high-risk HPV types. Moreover, it was reported that disulfide bound reduction therapies are related with immune response [25, 26]. Therefore, increase in disulfide level as dysplastic lesion progresses may be an answer for how the host response to virus infection occurs. According to this data, the disulfide value may be useful in determining whether HPV is low risk or high risk. Moreover, in future, possible disulfide reducing therapies to combat this oxidative stress in patients with LSIL or HSIL may come into question.

IMA has been presented as a new oxidative stress marker due to mainly on ischemic conditions [27]. As the amount of hydroxyl free radical increases in the environment, the N-terminus in the albumin skeleton is damaged. Therefore, while the level of albumin in circulation decreases, the level of IMA increases [28]. In fact, the conversion of normal albumin to IMA occurs with the addition of hypooxygenization (hypoxic media) to the low pH medium. Marcela et al. stated in their study that hypoxia inducible factor-1 is expressed weakly in HSIL and strongly in cervical cancer. It has been reported that direct hypoxia is also required as well as angiogenetic factors for angiogenesis in the early stages of malignant transformation [29]. IMA has never been studied in preinvasive cervical lesions before and we have not found any data to compare it with in the literature. Our study showed that albumin levels dropped and IMA values rose significantly as the lesion progressed. These data suggest that hypoxia and ischemia may be effective in increasing the degree of cervical preinvasive lesions.

In their study of premalignant lesions of the cervix, Batmaz and colleagues found that serum TOC and OSI values increased as they progressed from the control group to HSIL [30]. In the current research, we did not find significant difference in TAC and TOC values, but the OSI value was found to be significantly higher in preinvasive lesions. According to these data, we cannot say that TAC and TOC values can be used to distinguish cervical preinvasive lesions. However, the OSI value may contribute to further studies in oxidative stress assessments.

Our study has several limitations. One of the limitations of our analysis includes the lack of comparison with cervical cancer patients. The other limitation was the absence of longitudinal patient follow-up to observe the changes in the thiol/disulfide homeostasis parameters and other oxidative stress parameters during the follow-up period. Nevertheless, the prospective study design and the homogeneity of the characteristics of our study cohort were the major strengths of the present research. Prospective cohort studies including cervical cancer patients with LSIL and HSIL are still warranted to confirm our findings.

## Conclusion

The analysis of dynamic TD balance revealed considerable oxidative damage in patients with HPV-related cervical precursor lesions compared to women with ordinary cytology

specimens. This indicates that oxidative damage is dependent on the viral infection. These findings address that deterioration of dynamic TD balance may be a factor underlying the etiopathogenesis of cervical preinvasive lesions and may be related to HPV type. Therefore, investigation of TD balance with presented method in our daily practice represents a new promising test for early diagnosis (e.g., strict follow-up strategies) and management of women at high risk for cervical cancer.

**Author's contributions** BS: project administration, writing—reviewing and editing; FP: data curation, methodology, and investigation; AC and EAŞ: visualization, writing—original draft preparation; ÖE and SN: formal analysis; HC: conceptualization, supervision, and validation.

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## Declarations

**Conflict of interest** B.Sezgin, Fatih Pirinççi, A.Camuzcuoğlu, Eda Adeviye Şahin, Özcan Erel, Salim Neşelioğlu and H.Camuzcuoğlu declare that they have no conflict of interests.

**Ethical approval** Institutional review board approval for this research was obtained from Mugla Sitki Kocman University Medical Ethics Committee (Approval date and number: 19/09/2018-03). The trial was registered at ClinicalTrials.gov (ID: NCT04177641). An informed consent following the declaration of Helsinki was signed and obtained from the participants.

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