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Iron Indices and Urinary 8-Oxo-7, 8-Dihydro-2'-Deoxyguanosine (8-Oxodg) in Patients with Cervical Intraepithelial Neoplasia

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FAF and JIA designed the study, authors FAF and KOA recruited the patients and collected samples. Author OSI performed the statistical analysis, authors FAF, MDE, PMWL and VM analyzed the blood and urine samples and authors FAF, JIA and KOA participated in writing of the manuscript, author AAO contributed to the design of the protocol. Author FAF managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To investigate the role of iron status in cervical carcinogenesis through its involvement in the Haber-Weis and Fenton reactions serving as a pathway to carcinogenesis and using 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) as a marker of DNA oxidation in a population where iron deficiency is prevalent.
Study Design: It is a cross sectional study.
Place of Study: The patients were recruited from the colposcopy clinic of the University College Hospital (UCH), Ibadan and Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife, Nigeria. The laboratory investigations were carried out at the Haematology and Chemical Pathology laboratories of UCH, Ibadan and Oxidative Stress Group, Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, UK.
Methodology: Forty-five subjects with CIN and 41 with normal Pap smear result (non-CIN) were recruited. A structured questionnaire was administered to collect information on demographic characteristics, dietary, social and medical history. Fasting blood sample were collected to assess for serum iron, total iron binding capacity and transferrin saturation. Urine was also collected to analyze for creatinine and 8-oxodG.
Results: The CIN subjects had more babies; > 5 than non-CIN subjects (P=.003). The individuals with > 5 children were 4 times more likely to have CIN [OR 3.79 (95% CI 1.3-10.33), P=.01]. CIN subjects had higher serum iron and transferrin saturation than non-CIN subjects. Though the mean urinary 8-oxodG level similar between the two groups, there was a trend towards higher levels in individuals with high grade CIN.
Conclusion: High serum iron level was linked to frequent ingestion of iron supplement and may contribute to progression of CIN with a potential role for urinary 8-oxodG as a useful bio indicator of altered iron homeostasis and associated DNA damage.

Keywords: Iron; cervical intraepithelial neoplasia; DNA oxidation; 8-oxo-7; 8-dihydro-2'-deoxyguanosine (8-oxodG).

1. INTRODUCTION

Iron (Fe) is the most abundant metal in the human body, it is an essential nutrient, but when inappropriately stored, could be involved in free-radical generation [1,2]. Iron may participate in the pathogenesis of cancer in several ways, one of which is catalysis of the Haber –Weiss reaction and participation in the Fenton reaction, that generate reactive oxygen species (ROS) which could cause oxidation of biomolecules including nucleic acids (DNA, RNA), lipids and proteins, leading to pathological disturbance in cellular functions [1,3]. The redox properties of iron make it a potentially important participant in free radical production, when stored inappropriately, for example when body stores are saturated or disturbed, leading to tissue damage, mutation and carcinogenesis [1,2,4]. That iron can induce malignant transformation was first reported in 1959 by repeated intramuscular injection of iron dextran [5]. This has been further demonstrated in animal models and epidemiological studies [6,7,8]. The involvement of iron in colorectal and liver cancers has been extensively studied [3], including its implication in breast and endometrial cancer [9,10]. Other oncogenic effects of iron, besides a proposed role in production of ROS and other free radicals, include induction of oxidant-responsive transcription factors and nutrient support of cell growth [2,3]. Increased body iron stores have been implicated in cervical carcinogenesis [11,12].

Cervical cancer is the second most common cancer among women in developing countries [13,14] and the most common gynaecological cancer in Nigeria. The precursor lesion from which invasive cervical cancer develops is Cervical Intraepithelial Neoplasia (CIN). The development of vaccine against human papilloma virus (HPV) is a major milestone to prevent cervical cancer, however a vaccine is not universally available to individuals in many developing countries and at present is prohibitively expensive for a large percentage of these populations. Other approaches to reduce CIN which could later transform to cervical cancer are needed; therefore this study was carried out to assess the possible relationship between iron and cervical carcinogenesis.

It is proposed in this study that iron overload may exist in women with attendant disorders of iron homeostasis and consequent increase in free iron. Iron overload could also occur by non-selective administration of iron during periods of high demand such as pregnancy or during self-medication for symptoms mimicking anaemia [15,16]. Though iron deficiency is prevalent in many developing countries, one cannot completely rule out a possibility of recourse to self- medication with iron resulting in elevated body iron stores. In addition to the independent role of iron as a pro-oxidant, high levels of free iron may represent a host factor that can potentiate the mutagenic actions of HPV a known cause of cervical cancer. Oxidative stress arising from the above pathobiological pathway, as it impacts on DNA or dGTP pools could be assessed by the determination of 8-oxo-7, 8-dihydro-2'deoxyguanosine (8-oxodG), a marker of oxidation of DNA or dGTP excreted into urine. This study aims to investigate the role of iron status in cervical carcinogenesis through its involvement in the Haber-Weis and Fenton reactions serving as a pathway to carcinogenesis and using 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) as a marker of DNA oxidation in a population where iron deficiency is prevalent.

2. MATERIALS AND METHODS

2.1 Subjects and Sample Collection

A total of 86 women aged 26 to 71 years who attended the colposcopy clinic of the University College Hospital (UCH), Ibadan and Obafemi Awolowo University Teaching Hospitals Complex (OAUTH), Ile-Ife, Nigeria and gave consent to participate were enrolled into the study. Women on treatment for other diseases were excluded from the study. A structured questionnaire was administered to each participant to collect information on demographic characteristics, dietary, social and medical history. Data collection was by personal interview using either English or a version of the questionnaire based on a participant's native language of communication. Fasting blood samples were obtained at the time of collection of Pap smear results. Five millilitres of blood were collected into an iron-free plain bottle for iron studies. A spot urine sample was collected from each participant at the time of venipuncture into a universal bottle to assess the level of 8-oxodG and urinary creatinine (to correct for urine concentration). Blood samples in the plain bottles were allowed to clot. The serum sample from each subject was stored at -20ºC until analysis for serum iron studies.

2.2 Iron Analysis

Teco Diagnostics iron/TIBC reagent set (Teco Diagnostics, ANAHEIM, CA, USA) was used for determination of serum iron level and total iron binding capacity (TIBC). Transferrin saturation was derived by calculation using serum iron and TIBC values obtained by analyzing the serum.

Transferrin saturation (%) = (100 x serum iron)/TIBC

All analyses were carried out according to manufacturer's instructions

2.3 Analysis of Urinary 8-oxodG

Analysis of urinary 8-oxodG was performed by a UPLC-MS/MS procedure in the Oxidative Stress Group, Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, UK, using a procedure described by Lam et al. [17] and adapted for application to urinary 8-oxodG analysis. Briefly the procedure was carried out a follows:

Solid-Phase extraction: A 50 mg ENV+ ENVisolute SPE column was pre-conditioned with methanol and ultrapure water prior to application of 0.5 mL particulate-free urine, containing 10 pmol [15N5]-8-oxodG internal standard. Urine was drawn under vacuum and the column bed washed with two aliquots of ultrapure water. Bound material was eluted with two aliquots of 20% acetontirile in methanol. The eluent was dried under a nitrogen stream, then re-constituted in ultrapure water and transferred to UPLC autosampler vials.

UPLC-MS/MS: The UPLC-ESI-MS/MS system comprised an Acquity UPLC in line with a Quattro Premier tandem mass spectrometer (Waters, Elstree, UK). The UPLC conditions were as follows: column - an Acquity UPLC BEH C18 (2.1 x 100mm); mobile phase: 5% v/v methanol in water containing 0.1% formic acid, flow rate of 0.4ml/min; chromatograpy performed at 40ºC; samples were maintained at 4ºC throughout. Analytes were quantified using tandem electrospray mass spectrometry in positive ion mode (ES+). Product ions were monitored in multiple reaction monitoring mode using the mass transitions for 8-oxo-dG (m/z 284>167.9) and [15N5]-8-oxo-dG (m/z 289>173). Injection volumes for samples and standards were 5 μl. Peaks were integrated using Masslynx

software version 4.1. The limit of detection and reproducibility data for this method are reported in the article by Lam et al. [17].

The level of 8-oxodG in each urine sample was determined from the ratio of the peak area of 8-oxodG to that of the internal standard. The 8-oxodG level was corrected for urine concentration using creatinine. Aliquots of urine were also assayed for creatinine using the Jaffe method (Fortress Diagnostics Limited, Antrim, Northern Ireland, UK) in the Department of Chemical Pathology, University College Hospital, Ibadan. Urinary measurements of 8-oxodG were corrected accordingly, to give 8-oxodG pmol/µmol creatinine.

2.4 Statistical Analysis

Data were analyzed using the SPSS version 21. The student's t-test was used to compare means of continuous variables that were normally distributed. The median of skewed continuous variables were analyzed using Man-Whitney U and Kruskal-Wallis test for two or three medians respectively. For the purpose of this study, iron indices were categorized into low (<9.0 µmol/L), normal (9.0-30.4 µmol/L) and high (>30.4 µmol/L) values [18]. Also in line with these categories, the TIBC values were categorized as low (< 8.03 µmol/L), normal (8.03-12.83 µmol/L) and high (>12.83 µmol/L), as were transferrin saturation values, low (<16%), normal (16-50%) and high (>50%) (14). The 8-oxodG levels were categorized into three <0.5 pmol/µmol creatinine, 0.5-4.2 pmol/µmol cr and > 4.2 pmol/µmol cr [17]. CIN subjects were categorized according to progressive stages of dysplasia CIN I was categorized as low risk while CIN II and CIN III were categorized as high risk. Inferential statistics using Chi-square test was used to compare selected risk factors, iron ingestion habit with presence or absence of CIN.

Variables with possibility of predicting CIN were used in the logistic regression model while adjusting for sexual partners and number of delivery to determine the factors predicting the presence of CIN. Logistic regression results were reported using odds ratio and 95% Confidence Interval. Level of statistical significance was set at $P < .05$.

3. RESULTS AND DISCUSSION

The mean age for women with CIN was 47±9 years, while for non-CIN was 45±10 years. The difference in age between CIN and non-CIN was not statistically significant. The pattern of meat ingestion (quantified by asking for the portion of red meat consumed), a major source of iron, was comparable between both groups of subjects. Use of iron supplements in the CIN group was more during pregnancy, though not significant statistically (Table 1).

Use of iron supplementation during pregnancy 19 (50.0%) in CIN and 14 (35.0%) in no CIN but p-value=0.18. There was a statistically significant difference in the number of deliveries between women with CIN and those without CIN; women with CIN were more likely to have five or more deliveries than women without CIN (P=.003). Hormonal therapy as the mode of contraception was used in 20.0% of CIN subjects compared to 9.8% of non-CIN subjects (P=.07).

The distribution of the values of urinary 8-oxodG level in patients with CIN and non-CIN were almost similar (Fig. 1).

Higher serum iron and transferrin saturation were more frequently observed in CIN patients (Figs. 2 and 3).

Eleven subjects had serum iron level greater than 30.4 µmol/L, of which 7 (15.6%) had CIN and 4 of them did not have CIN. Five participants had transferrin saturation above 50%; four of these had CIN. There was a statistically significant higher TIBC level in subjects with CIN compared to subjects without CIN (P=.02) while the differences observed in the median levels of serum iron and transferrin saturation in both groups were not statistically significantly different (Table 2).

There were 21 women with low grade and 24 with high grade CIN. Comparison of median serum iron (SI) level, total iron binding capacity (TIBC), transferrin saturation (Trf. Sat), and 8-oxodG levels between the two categories of CIN showed that women did not show statistically significance in the differences seen (Table 3).

Individuals with 5 children and above are about 4 times more likely to have CIN [OR 3.9 (95% CI 1.4-10.7) P = .01]. Controlling for number of sexual partners and parity, the odds for CIN among those with transferrin saturation greater than 50% is 5.8 compared to 2.2 among those with transferrin saturation less than 16%.

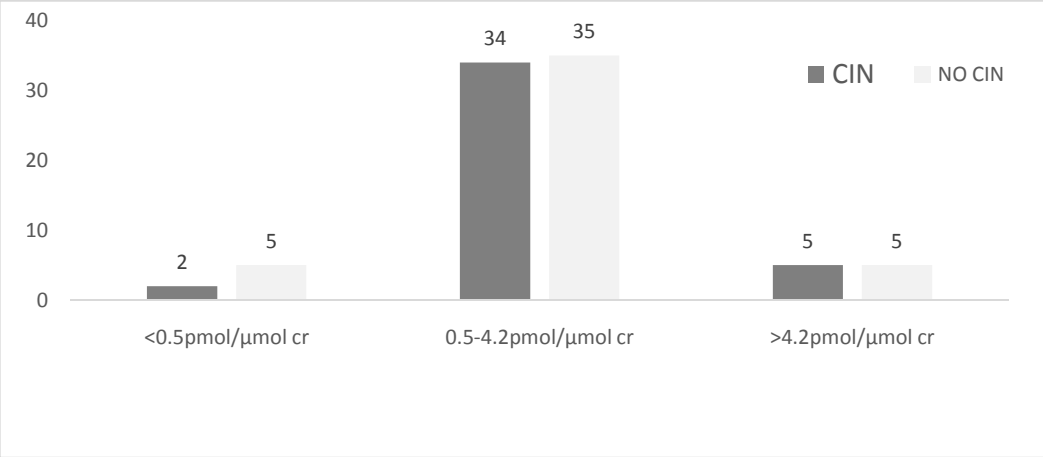

Fig. 1. Distribution of 8-oxodG in those with CIN and those without CIN

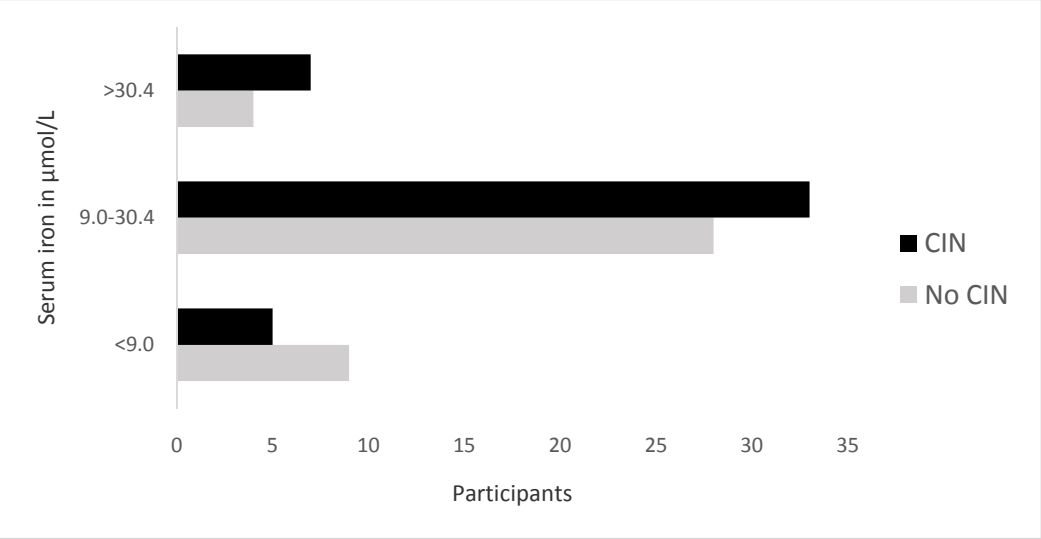

Fig. 2. Serum Iron in those with and without CIN

3.1 Discussion

Cervical carcinogenesis is a multistep process. Invasive cervical cancer is preceded by CIN that is almost always associated with HPV infection [19,20]. However, these lesions and the viral infection may spontaneously regress or progress. There is no way of knowing the factors that enhance the regression or progression of HPV infections and CIN. These have prompted the search for other factors or co-factors that could promote cervical carcinogenesis. Studies have linked raised iron stores to cervical carcinogenesis [21,22]. In this study, the serum iron and transferrin saturation, indicators of iron burden were higher in subjects with CIN compared to subjects without CIN, though not statistically significant. The higher serum iron level could encourage a perturbed iron homeostasis with increased trafficking and level of bioavailable iron in cervical epithelial cells [23]. This is further implied by the higher odds for CIN with elevated serum iron and transferrin saturation.

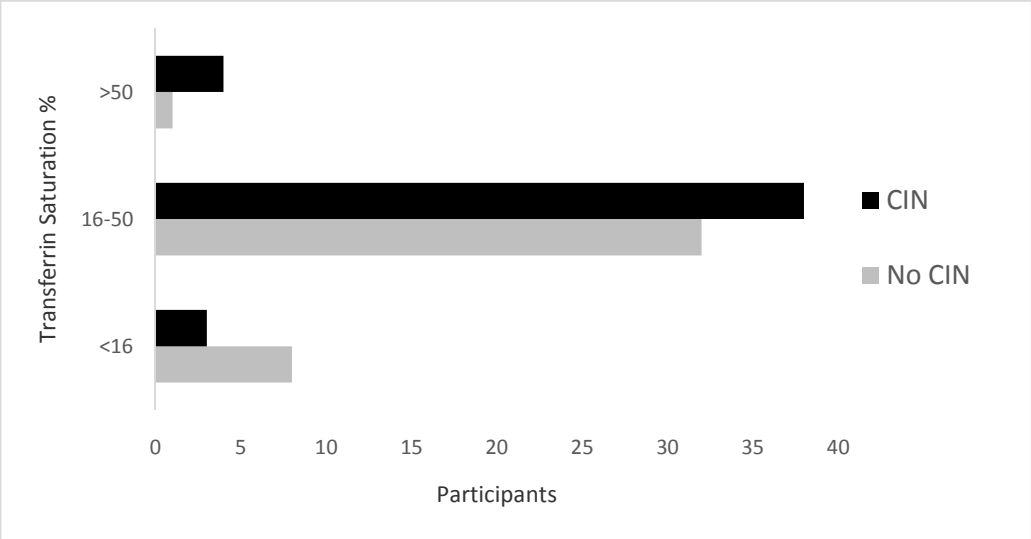

Fig. 3. Transferrin saturation in those with and without CIN

Table 1. Risk factors for CIN and iron ingestion habits of study population

Variables	CIN		Chi-square	*P*-Value
	Present	Absent		
Educational attainment				
Less than high school	12 (26.7)	6 (14.6)	1.877	.17
Completed high school	33 (73.3)	35 (85.4)		
Ever smoke cigarette				
Yes	1(2.2)	0 (0)	0.922	.337
No	44 (97.8)	41(100)		
Alcohol consumption				
Yes	10 (22.2)	5 (12.2)	1.498	.221
No	35 (77.8)	36 (87.8)		
Parity				
<5	15(33.33)	27 (65.9)	9.080	.003
≥5	30(66.7)	14 (34.1)		
Live time sexual partner				
1	31(68.9)	26 (63.4)	0.228	.592
>1	14(31.1)	15 (36.6)		
Hormonal contraception				
Ever use	9 (20.0)	4(9.8)	3.381	.07
Never use	36 (80.0)	37(90.2)		
Menopausal status				
Menopausal	16 (35.6)	11(26.8)	0.766	.682
Peri-menopausal	9 (20.0)	9 (22.0)		
Menstruating	20 (44.4)	21(51.2)		
Use of Iron supplement				
During pregnancy	19 (50.0)	14 (35.0)	1.796	.180
Not during pregnancy (daily/occasionally)	19 (50.0)	26 (65.0)		
Meat intake				
Daily	17 (37.8)	17 (41.5)	0.152	.927
1-3weeks	17 (37.8)	14 (34.1)		
When available	11 (24.4)	10 (24.4)		

Table 2. Iron studies and haematological profile of the study population

Variables	CIN		*P*-value
	Present	Absent	
Iron Studies*	Median(Range)	Median(Range)	
Iron (µmol/L)	16.97 (4.6-77.2)	13.89(3.1-77.2)	.07
TIBC (µmol/L)	73.6 (12.0-142.0)	67.6(22.4-135.2)	.02
Transferrin saturation (%)	30(12.0-78.0)	24(5-57.0)	.195
8-oxodG (pmol/mg cr)	1.5(0.1-9.1)	1.9(0.2-9.6)	.398

**Man-whitney U test*

Table 3. Comparison of iron indices and 8-oxodG levels in low and high risk CIN stages

CIN stage	N	Serum iron (SI) µmol/L median (range)	Total Iron binding capacity (TIBC) µmol/L median (range)	Transferrin saturation (Trf Sat) % median (range)	8-OxodG pmol/µmol cr median (range)
Low risk	21	15.4 (4.6-38.6)	72.1 (20.0-98.4)	31 (12.0-78.0)	1.1 (0.1-9.1)
High risk	24	20.1 (7.7-77.2)	75.6 (11.9-142.0)	30 (15.0-64.3)	1.9 (0.4-6.8)
P-value		.291	.291	.889	.098

The oxidant effect of iron overload could not be demonstrated in this study in view of the similar level of 8-oxodG in both CIN and non-CIN groups. This may be because iron is not the only determinant of formation and excretion of 8-oxodG. Amongst the products of DNA oxidation, 8-oxoguanine is the most important with the potential to induce point mutation in genomic DNA, similarly the dGTP pool is a vulnerable target for ROS with the potential for 8-oxodGTP to be mis-incorporated into DNA, again to induce point mutations [24,25]. The nucleotide pool sanitizing actions of NUDT1, 15 and 18 can yield 8-oxodG possibly contributing to urinary 8-oxodG levels [26]. The relatively large number of reports measuring urinary 8-oxodG as a biomarker of oxidative stress and DNA damage also prompted the use of 8-oxodG to assess the role of iron status in oxidative modification of DNA and its synthetic precursors [24,25,27]. The link between 8-oxodG, oxidative damage to DNA and progression of cervical dysplasia is suggested by a study by Hirahu et al. [28] and other studies [29] which could be the consequence of an unknown non-HPV event. A trend towards increasing 8-oxodG with progressive disease was observed, though not statistically significant, but should not be completely ignored and may suggest some degree of oxidative DNA damage. However iron as nutrient for the cells should be considered [3].

Most studies linking iron status to cancer were done in North America and European countries where iron supplements and iron-fortified foods are widely used [7,8,9,12]. In contrast, this study was conducted in a region where iron fortified food is not generally used and iron deficiency is prevalent. These peculiarities could also be responsible for the lack of significant differences in iron burden between subjects with CIN and those without CIN, consequently the product of oxidative damage. A study conducted in France where iron supplementation and iron fortified foods are not widely used suggested that the relationship between iron and risk of cancer is a controversial issue [30], a finding compounded by observations of an inverse relationship between iron status and cancer [31], or no apparent relationship [32].

The higher number of parity and the use of iron supplements in pregnancy is likely to be responsible for a relatively higher serum iron level in the CIN group. The difference in TIBC between the two groups reflects the confounding factors associated with both the development of CIN and physiological changes which may influence iron transport (transferrin). The significantly higher TIBC observed in the CIN patients might have resulted from hormonal contraceptive use and pregnancy [33] known to increase transferrin synthesis and raise the total iron binding capacity. The use of hormonal contraception and higher parity means partners are less likely to use condoms, therefore the risk of HPV exposure is greater. Antenatal iron supplements consumed daily has been reported to produce oxidative stress [15,16] possibly due to de-compartmentalized non-transferrin bound iron.

There are suggestions that markers of increased iron burden commonly used in epidemiological studies, such as serum ferritin, transferrin saturation, serum iron or iron binding capacity may be inappropriate indicators to investigate the harmful health effects of iron overload [34,3]. Substances that contain iron bound in redox active form in the body are transient and difficult to detect. The, non-transferrin bound iron better indicators of redox active iron, this can be assessed using calcein as fluorescent probe for low molecular weight iron [3]. Research has shown that minimal toxic concentration of iron varies with cell type and disparity in iron susceptibility might arise from differing capacities to synthesize antioxidants or ferritin [34,35]. Therefore, iron-catalysed oxidative damage may be observed in some tissues despite normal transferrin saturation levels [36].

4. CONCLUSION

Women with CIN had relatively higher serum iron when compared with subjects without CIN, however, this did not reach statistical significance. The urinary 8-oxodG was similar for both CIN and non-CIN. The higher level of urinary 8-oxodG observed in the high risk group may suggest the need for a larger multi centre studies in order to shed more light on the links between excessive body iron, urinary 8-oxodG, cervical pre-malignant lesions and the risk of developing cervical cancer.

ETHICAL APPROVAL

Ethical approval was obtained from the University of Ibadan/University College Hospital and OAUTH, Ile-Ife Ethical Review Committees

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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