Female Reproductive Hormones and Biomarkers of Oxidative Stress in Genital Chlamydia Infection in Tubal Factor Infertility

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Abstract

Background: Genital Chlamydia infection (GCI) and the associated pathologies have been implicated in tubal infertility. Though the actual pathologic mechanisms are still uncertain, oxidative stress and other factors have been implicated. The purpose of the study was to determine the possible contribution of female reproductive hormones and biomarkers of oxidative stress in genital Chlamydial infection to tubal occlusion.

Methods: This prospective case control study was carried out by recruiting 150 age matched women grouped into infertile Chlamydia positive women (n=50), fertile Chlamydia positive women (n=50) and fertile Chlamydia negative women as controls (n=50). High vaginal swabs and endocervical swabs were collected for screening *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, *Staphylococcus aureus*, and *Candida albicans*. Sera were collected for estimation of *Chlamydia trachomatis* antibody, female reproductive hormones [Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Oestradiol (E_2), Progesterone (P_4), Prolactin (PRL)] and biomarkers of oxidative stress [Total Antioxidant Capacity (TAC) and 8-hydroxyl-2-deoxyguanosine (8-OHdG)] by enzyme immunoassay (EIA). Data were analyzed using chi square, analysis of variance and LSD Post hoc to determine mean differences at p=0.05.

Results: Among women with GCI, higher levels of LH and 8-OHdG were observed in infertile Chlamydia positive women compared to fertile Chlamydia positive women (p<0.05). Higher levels of LH and 8-OHdG and lower TAC levels were observed in infertile Chlamydia positive women compared to fertile Chlamydia negative controls (p<0.05).

Conclusion: Mechanisms including oxidative DNA damage and reduced antioxidant capacity may be involved in the pathology of Chlamydia induced tubal damage.

Introduction

Genital Chlamydia infection is considered the most prevalent sexually transmitted bacterial infection throughout the world. It has been described as potentially, the major cause of tubal occlusion and tubal infertility in women (1). The exact pathologic mechanism of Chlamydial induced tubal damage has not been elucidated. However, factors such as human genetics (2) and en-

docrinology (3), cytokine profile (4), previous infections (5), pathogen load (6, 7), Chlamydial strain, presence of other genital infections (1, 7), route of infection (8), oxidative stress and pathologic immune responses to the bacterium have been implicated (9). In all cases, however, the pathology seems to be related to a chronic inflammation caused by host immune response to chronic persistent Chlamydial infection or frequent reinfections with the bacterium (10).

Inflammatory response to genital Chlamydia infection leads to activation of polymorphonuclear leukocytes and macrophages resulting in increased production of reactive oxygen species (ROS) (11). Production of ROS in excess of antioxidants results in oxidative stress. Oxidative stress precipitates a range of pathologic changes that affect the reproductive functions in both men and women (12). It has been implicated in the pathophysiology of tubal factor infertility, endometriosis, preeclampsia, free radical induced birth defects and abortions (13-16). Postulated pathologic mechanisms include oxidative DNA damage, lipid peroxidation, modulation of gene expression and transcription factors, inhibition of protein synthesis, and depletion of ATP (17). Biomarkers of oxidative stress have been localized in various sites of female reproductive tract (18) and elevated ROS levels have been demonstrated in idiopathic infertility (16).

The female sex hormones have been reported to exert a number of effects on the immunity and clinical outcome of Chlamydial infections of the female genital tract (19). Studies using animal models of genital tract Chlamydial infection suggest that the hormonal status of the genital tract epithelium at the time of exposure may influence the outcome of infection (20). The mechanisms by which sex steroids orchestrate the balance between reproduction and protection against pathogenic microorganisms, however, are poorly understood. Understanding the role of sex steroids in genital Chlamydial infection may be important in preventing the development of sequelae.

Serum levels of female reproductive hormones [Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Oestradiol (E$_2$), Progesterone (P$_4$), Prolactin (PRL)] and oxidative stress biomarkers [Total Antioxidant Capacity (TAC) and 8-hydroxyl-2-deoxyguanosine (8-OHdG)] were estimated in infertile Chlamydia positive women (tubal infertility), fertile Chlamydia positive women and fertile Chlamydia negative women to determine their possible contribution to tubal occlusion in genital Chlamydial infection.

Methods

Study Design: The study was a prospective case control study conducted in the Gynaecology and Family planning Clinics of the Department of Obstetrics and Gynaecology, University College Hospital and Adeoyo Maternity Hospital, Ibadan, Nigeria in the years 2009 to 2010. Study protocol was approved by the University of Ibadan/ University College Hospital Ethics Committee reference number UI/EC/08/0083. Informed consent was obtained from the subjects before recruitment into the study.

Inclusion criteria were subjects with infertility for at least one year duration, child birth of less than 2 years for the fertile controls and subjects that gave consent. Exclusion criteria were subjects with previous history of uterine surgery, subjects undergoing any form of contraceptive therapy, malignancy, long term medication and chronic or systemic illness and those that did not give consent. Infertility was defined in this study as the inability of a couple to conceive after a period of 12 months of unprotected intercourse (21). Evidence of fertility was taken as ability to have at least one child, with the last childbirth within the last 2 years. Evaluation of infertility was carried out using standard procedures according to National Health Service evaluation criteria (22).

Socio-demographic characteristics of the study population-family history, social history, past medical history, medication and gynaecological history were obtained using a semi-structured questionnaire. Anthropometric indices-height, weight, hip and waist circumference were taken to calculate the body mass index and waist to hip ratio, respectively. Subjects were screened for the presence of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, *Staphylococcus aureus* and *Candida albicans* using standard methods (23). These women were further screened for presence of *C. trachomatis* antibodies (CT IgG).

Selection of Subjects: A total of 150 age matched women of reproductive age (100 fertile and 50 with tubal infertility) without microbial antigens (*Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, *Staphylococcus aureus* and *Candida albicans*) were consecutively recruited into this prospective case control study. Bilateral tubal blockage was iden-

tified by hysterosalpingogram. These women were further sub-divided into 3 groups based on Chlamydia antibody positivity into infertile Chlamydia positive women (n=50), fertile Chlamydia positive women (n=50) and their corresponding fertile Chlamydia negative women as controls (n=50).

Sample collection: Ten milliliters of venous blood samples were collected aseptically from each subject on day 3 (follicular phase) of the menstrual cycle. An additional 5 *ml* of venous blood samples were collected from the study group only on days 21-23 (luteal phase) for progesterone estimation and assessment of ovulation. This is because the members in control group were recruited during their follicular phase and they would have been taking contraceptives by the time they were in the luteal phase, making them unsuitable for progesterone assay. Samples were dispensed into plain sample containers. After clot retraction, samples were centrifuged at 500 *g* for ten minutes after which serum was extracted and stored in small aliquots at -20°C until time of analysis. High vaginal swabs (HVS) and endocervical swabs (ECS) were collected from all subjects of study for isolation of such pathogens as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, *Staphylococcus aureus* and *Candida albicans* within one hour. Hormones [(-Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), Prolactin (PRL), Progesterone (P4) and Estradiol (E2)], biomarkers of oxidative stress [(Total Antioxidant Capacity (TAC), 8-hydoxy-2-deoxyguanosine (8-OHdG)] were analyzed in serum samples of the women of the study.

Detection of Chlamydia antigens was carried out by Immunochromatographic method using prepared test kits (Diaspots Diagnostics, USA) (24).

Isolation of Candida species and bacterial vaginosis was done by Gram stain procedure, (Oxoid chemicals, USA) (25). Identification of *Trichomonas vaginalis* was done by microscopy (26). Isolation of *Neisseria gonorrhoeae* was done by culture method using Thayer Martin culture medium (Becton, Dickinson, USA) (27). Quantification of Chlamydial antibodies (IgG) was carried out by enzyme immunoassay (EIA) method (Orgenics Ltd, Germany) (28). Detection of Treponema pallidum antibodies (IgG and IgM) was done by Immunochromatographic method (Acon diagnostics, USA) (29).

Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Prolactin (PRL), Estradiol

(E$_2$) and Progesterone (P$_4$) estimation was done by enzyme immunoassay (EIA) method (Immunometrics, UK) (30). Total Antioxidant Capacity (TAC) was estimated by Trolox Assay (Cayman Chemicals, USA) (31). Estimation of 8-hydroxy-2-deoxyguanosine (8-OHdG) was done by enzyme immunoassay (Cayman Chemicals, USA) (32).

Statistical analysis: Data analysis was done using the statistical package for social sciences (SPSS version 20.0). Analysis of variance (ANOVA) was used to test significance of variations within and among group means. Fisher's least significant difference (LSD) test was used for comparison of multiple group means. Chi square analysis was used for comparison of means for non quantitative variables. A two sided p<0.05 was considered statistically significant.

Results

Table 1 shows the past history of symptoms of GTI, antibiotic therapy, alcohol use and smoking habits in fertile CT neg (*Chlamydia trachomatis* negative), fertile CT pos (*Chlamydia trachomatis* positive) and infertile CT pos women (tubal infertility). Significant differences were observed in the percentage of women with past history of vaginal itching, vaginal discharge, lower abdominal pain, current antibiotic therapy (<3 months) and smoking habit among controls and Chlamydia positive women. No significant difference was observed in alcohol consumption habit of the groups.

Table 2 shows mean age and anthropometric indices, serum levels of hormones (FSH, LH, PRL and E$_2$) and oxidative stress markers (8-OHdG and TAC) in fertile CT neg, fertile CT pos and infertile CT pos women (tubal infertility). Significant variations were observed in the serum levels of LH and 8-OHdG among the groups. No significant variations were observed in the mean age, anthropometric indices, FSH, PRL and E$_2$ among the groups. CT pos women with tubal infertility had normal P$_4$ levels indicating normal ovulatory cycles.

Table 3 shows comparisons of LH, 8-OHdG and TAC in fertile CT neg, fertile CT pos and infertile CT pos women (tubal infertility) using Post hoc analysis. Comparison of the parameters infertile CT neg and CT pos women with tubal infertility using Post hoc test showed significantly lower TAC levels and higher LH and 8-OHdG levels in infertile CT pos women with tubal infertility compared to fertile CT neg controls. No signifi-

Table 1. Past history of symptoms of GTI, antibiotic therapy, alcohol use and smoking habits in fertile CT neg, fertile CT pos, and infertile CT pos women (tubal infertility)

Symptom	Fertile CT neg n=50	Fertile CT pos n=50	Infertile CT pos n=50	p-value [*]
Vaginal discharge				
Yes	10(13.7%)	18(24.7%)	45(61.6%)	<0.001
No	40(51.9%)	32(41.6%)	5(6.5%)	
Vaginal itching				
Yes	7(9.6%)	16(21.9%)	50(68.5%)	<0.001
No	43(55.8%)	34(44.2%)	0(0.0%)	
Lower abd. pain				
Yes	3(13.0%)	6(26.1%)	14(60.1%)	0.007
No	47(37.0%)	34(44.6%)	36(28.3%)	
A/biotics (<3 months)				
Yes	16(19.8%)	22(27.2%)	43(53.1%)	<0.001
No	34(49.3%)	28(40.6%)	7(10.61%)	
Alcohol consumption				
>0 *g/day*	6(37.5%)	5(31.2%)	5(31.2%)	0.932
0 *g/day*	44(32.8%)	45(33.6%)	45(33.6%)	
Past smokers				
Yes	1(6.7%)	4(26.7%)	10(66.7%)	0.009
No	49(36.3%)	46(34.1%)	40(29.6%)	

Values are in number of subjects with percentage in parenthesis, ∗ Chi square test
GTI= Genital Tract Infection; CT pos= *Chlamydia Trachomatis* Positive, CT neg= *Chlamydia Trachomatis* Negative, Abd.=Abdominal, A/biotics=Antibiotics

Table 2. Age, anthropometric indices, hormones, oxidative stress markers in fertile CT neg, fertile CT pos and infertile CT pos women (tubal infertility)

Parameter	Fertile CT neg n=50	Fertile CT pos n=50	Infertile CT pos n=50	p-value [*]
Age (year)	34.90±0.46	33.94±0.48	33.76±0.51	0.351
Anthropometry				
Weight (*kg*)	68.60±2.19	64.92±1.83	69.60±1.38	0.249
Height (*m*)	1.59±0.07	1.5±0.01	1.60±0.01	0.465
BMI (kg/m^2)	26.90±0.78	25.87±0.65	27.01±0.49	0.548
WC (*cm*)	86.34±1.84	83.40±1.65	86.64±1.19	0.323
HC (*cm*)	103.94±1.72	102.76±1.71	104.32±1.18	0.895
WHR (*cm*)	0.83±0.07	0.81±0.07	0.83±0.06	0.125
Hormones				
FSH (*IU/L*)	9.09±0.79	8.50±0.51	9.67±0.2	0.547
LH (*IU/L*)	5.14±0.42	5.10±0.41	11.04±0.54	<0.001
PRL (*mIU/L*)	547.82±104.09	490.20±77.61	449.03±21.92	0.235
P_4 (*pmol/l*)	†	†	20.74±2.05	
E_2 (*pmol/L*)	0.49±0.05	0.46±0.04	1.01±0.46	0.352
OS markers				
8-OHdG (*pg/ml*)	1086.00±63.89	1004.0±62.12	1989.60±159.65	<0.001
TAC (*Mm*)	0.17±0.007	0.16±0.008	0.14±0.007	0.068

FSH= Follicle Stimulating Hormone, BMI= Body Mass Index, LH= Leutinizing Hormone, WC= Waist Circumference, PRL= Prolactin, HC= Hip Circumference, E_2= Estradiol, WHR= Waist to Hip Ratio, TAC= Total Antioxidant Capacity, OS= Oxidative Stress, 8-0HdG= 8-Hydroxy-2-Deoxyguanosine, P_4= Progesterone, CT pos= *Chlamydia Trachomatis* Positive, CT neg= *Chlamydia Trachomatis* Negative
† Luteal phase sample not collected (see sample collection), ∗ One way Anova

Table 3. Comparison of LH, 8-OHdG and TAC in fertile CT neg, fertile CT pos and infertile CT pos women (tubal infertility) using Post hoc analysis

Groups	Parameter	Mean Diff.	STD error	P-value
Fertile CT neg *vs.* infertile CT pos				
	LH (*IU/L*)	-5.90	0.694	<0.001
	8-OHdG (*pg/ml*)	-903.600	172.35	<0.001
	TAC (*Mm*)	-0.025	0.011	0.019
Fertile CT pos *vs.* infertile CT pos				
	LH (*IU/L*)	-5.94	0.694	<0.001
	8-OHdG (*pg/ml*)	-985.602	172.35	<0.01
	TAC (*Mm*)	-0.020	0.010	0.061

LH= Leutinizing Hormone, TAC= Total Antioxidant Capacity, 8-0HdG= 8-hydroxy-2-Deoxy-guanosine, CT pos= *Chlamydia Trachomatis* Positive, CT neg= *Chlamydia Trachomatis* Negative

cant differences were observed in the levels of other indices in the two groups. Comparison of the parameters in fertile CT pos women and infertile CT pos women with tubal infertility using Post hoc analysis showed that the LH and 8-OHdG levels were significantly higher in infertile CT pos women with tubal infertility compared to fertile CT pos women. No significant differences were observed in the levels of other indices in the two groups.

Discussion

In the present study, the possible contribution of female reproductive hormones and biomarkers of oxidative stress to the development of Chlamydia induced tubal pathology was examined in Chlamydia positive women with tubal factor infertility and fertile Chlamydia positive women with fertile Chlamydia negative women serving as controls. The present study has shown that higher percentages of Chlamydia positive women with or without tubal infertility with past history of vaginal itching, vaginal discharge and lower abdominal pain were observed compared to fertile Chlamydial negative controls (Table 1). Vaginal itching, vaginal discharge and lower abdominal pain have been described as the most important perceived symptoms of genital tract infection among women attending the family planning and gyneacology clinics in Lagos, Nigeria (33, 34). Female reproductive tract infections (RTIs) usually originate in the lower genital tract as vaginitis or cervicitis and may produce symptoms such as abnormal vaginal discharge, genital pain, itching and burning feeling with urination. Past Chlamydial infection may be responsible for higher prevalence of these symptoms in these women (33). The higher use of antibiotics in Chlamydia positive women with or without tubal infertility compared to fertile Chlamydial negative controls in this study suggests possible awareness of these women about the consequences of genital tract infections on fertility in Ibadan. Prescriptions and use of antibiotics without laboratory guidance as well as over the counter sale of antibiotics without prescription are common practices in Nigeria. Frequent use and abuse of antibiotics have been observed both in and out-patients of a tertiary hospital in Benin, Nigeria (35).

Significant variations in the levels of reproductive hormones and biomarkers of oxidative stress were observed among infertile Chlamydia positive women (with tubal infertility), fertile Chlamydia positive women and their corresponding fertile Chlamydia negative controls (p<0.05) (Table 2). Slightly elevated levels of LH, increased 8-OHdG and reduced TAC were observed in Chlamydia positive women with tubal infertility compared to the fertile Chlamydia negative controls (p<0.05) (Table 3). These observations suggest that elevated LH and 8-OHdG, and reduced TAC may either be involved in the pathologic mechanism of Chlamydia induced tubal damage or may be the consequence of the body's physiologic response to the presence of GCI. Association and biologic interactions between sex hormones, ROS and antioxidants have been reported in tubal infertility (36-38) and normal tubal secretory function has been correlated with low ROS concentrations (19). Infection of tubular epithelial cells by Chlamydia induces production of reactive oxygen species (11), and the consequent oxidative stress (OS) can cause oxidative DNA damage, which may result in higher levels of 8-OHdG as seen in Chlamydia positive women with tubal infertility in this study. 8-hydroxy-2-deoxyganosine (8-OHdG), a

biomarker for endogenous oxidative DNA damage, has been implicated in the development of benign gynaecological conditions (39) and elevated levels have been associated with lower fertilization rates and poor oocyte quality (40). Significantly higher levels of 8-OHdG have also been reported in immunochemical staining of biopsied tissues from tubal cyst compared to those from normal healthy fallopian tubes (39). *C. trachomatis* infection in several cell lines has been demonstrated to cause the release of ROS and lipid peroxidation products (41). Peroxidative damage to deoxyribonucleic acid bases and phosphodiester backbones leads to the formation of altered nitrogenous bases, which affects replication and transcription processes leading to mutations and altered gene expressions (12, 42). The peroxidation could also cause membrane leakage that would eventually lead to cell lysis and allow spreading of Chlamydia elementary bodies. Surrounding cells may be peroxidized by the released ROS, which could partially account for the inflammation and cell damage observed during chlamydial infection (43). However, a study by Nsonwu-Anyanwu et al. (44) showed no significant differences in levels of total antioxidant potential (TAP), total plasma peroxidase (TPP) and oxidative stress index (OSI) between fertile Chlamydia negative women and Chlamydia positive women with or without tubal infertility.

Sex hormones have been shown to modulate immune responses and antioxidant functions in the female genital tract, and determine disease outcome (resolution or development of sequelae) (36, 38, 45, 46). LH has been described as an antioxidant hormone (47). It increases the antioxidant reserve of the corpus luteum leading to the maintenance of luteal functions and increased protection against the luteolytic actions of reactive oxygen species (48). LH also increases cell viability by increasing the mRNA and protein expression of antioxidant enzymes: Mn-SOD, Cu, Zn-SOD and catalase activity (49). It is also possible that mildly elevated LH levels in women with tubal infertility in this study exist in response to increased ROS induced oxidative DNA damage (elevated 8-OHdG).

Reduced TAC may imply elevated ROS and elevated ROS levels imply exhausted antioxidant defence, resulting in the inability to scavenge ROS and neutralize their toxic effects (50). Thus, lower TAC levels in genital Chlamydia positive infertile women, may imply increased susceptibility to ROS induced tubal damage in response to genital Chlamydia infection. Significantly lower antioxidant concentrations have also been demonstrated in the peritoneal fluid of patients with infertility compared with antioxidant levels in fertile patients (13).

No significant variations were observed in anthropometric indices (including BMI, WC, and WHR) among Chlamydia negative fertile controls and Chlamydia positive women with or without tubal infertility in this study, suggesting limited involvement of body fat and lipid peroxidation (p>0.05) (Table1).

Conclusion

This study has shown that mechanisms including increased Luteinizing Hormone, 8-hydroxy-2-deoxyguanosine and reduced TAC may be associated with and may probably mediate Chlamydia induced tubal damage. The link between decreased antioxidant status and lowered fecundity suggests a potential use for antioxidant supplementation as a prophylactic agent to avert ROS induced tubal pathology.

Acknowledgement

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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