**Investigating Oxidative Stress Levels in Pregnant Patients Infected with Hepatitis C Virus (HCV) and Bacterial Vaginosis (BV) for Better Treatment Option**

Muhammad Umer Asghar\(^1,2,3^*\), Kabeer Hanif\(^1,4\), Fizza Fatima\(^5\), Aisha Asghar\(^6\) and Noor Ul Ain\(^7^*\)

\(^1\)Institute of Microbiology (IOM), University of Agriculture Faisalabad (UAF), Faisalabad 38000, Pakistan.

\(^2\)National Institute for Biotechnology and Genetic engineering (NIBGE), Faisalabad 38000, Pakistan.

\(^3\)Pakistan Institute of Engineering and Applied Sciences (PIEAS), Nilor-Islamabad 45650, Pakistan.

\(^4\)School of Life Sciences, Institute for Immunology, Tsinghua University, Beijing 100084, China.

\(^5\)Rawalpindi Medical University, Rawalpindi 43600, Pakistan.

\(^6\)District Headquarters Hospital (DHQ), Toba Tek Singh 36050, Pakistan.

\(^7\)Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore 54590, Pakistan.

Received: 30 November 2023

Accepted: 19 March 2023

*Corresponding author: omer.asghar@yahoo.com; noor.phd.mmg@pu.edu.pk

**DOI 10.5001/omj.2023.102**

**Abstract**

**Objectives:** Hepatitis C virus and bacterial vaginosis coinfection generates sustained inflammation with bulk production of reactive oxygen species. They have the potency to cause hepatocellular carcinoma, vaginal apoptosis, disturbs the pregnancy, influence on drug treatment and follow up. The aim of this case-control study was to compare the redox status in Hepatitis C virus and bacterial vaginosis (coinfection) with respect to bacterial vaginosis (mono-infection) among pregnant females.

**Methods:** Blood samples and vaginal secretions were drawn from 75 pregnant females divided into three groups: coinfection (n=25), mono-infection (n=25) and control pregnant females (n=25) i.e., presumable healthy subjects. Blood samples were analyzed for hepatitis C virus detection based on conserved 5’ untranslated region via real-time PCR and hematological parameters were analyzed. Markers of oxidative stress (malondialdehyde, peroxidase) and antioxidants (catalase, superoxide dismutase) were checked in plasma as well as vaginal secretions of patients among all three groups.

**Results:** Hematological analysis reveals that hemoglobin level, platelets and lymphocytes decreased significantly (P<0.05) among coinfection followed by mono-infection group with respect to the control. Moreover, the higher isolation frequency of pathogenic bacteria (*Acinetobacter* spp.,) and nugent score trend was observed among coinfection group. Antioxidants levels were significantly lower (P<0.05) among the vaginal secretions and blood plasma of patients having co-infection with respect to the mono-infection and control group. While oxidative stress marker were significantly highest (P<0.05) among vaginal secretions and blood plasma of coinfection followed by mono-infection and control group. These results validate that overall redox severity was more among the coinfection as compared mono-infection and control pregnant females.
Conclusions: Redox indexes should be considered in early diagnosis and treatment of hepatitis C virus and bacterial vaginosis coinfection which may also facilitate the better treatment of hepatocellular carcinoma and vaginal apoptosis.

Keywords: Coinfection, Pregnant females, Oxidative stress, Hepatitis C Virus (HCV), Bacterial Vaginosis (BV)

Introduction

HCV belongs to Flaviviridae family a positive-sense RNA virus [1] that leads toward hepatocellular carcinoma with 3% worldwide prevalence and reported to be a vertically transmitted virus [2]. Pakistan, where health standards and living status of 170 million peoples are below average [1]. According to an estimate, 10 million people in Pakistan are positive for HCV virus [3]. Pathogenesis of HCV is based on biomarkers of oxidative stress i.e., reactive oxygen species (ROS) and reactive nitrogen species (RNS). Proteins of HCV induces the production of free radicals in hepatocytes in the form of lipid peroxidation and oxidized thioredoxin [4]. Moreover the excessive iron uptake causes the iron deposits in liver to react with ROS and produce more free radicals [5]. The body regulates the biomarkers of oxidative stress with reductants and oxidative enzymes. During oxidative stress, level of antioxidant enzymes get increased to counter the stress [5].

Bacterial vaginosis (BV) is a lower reproductive tract abnormal condition. It is causing the considerable public health expenditures and emotional distress in females [6]. Factors like hormonal fluctuation, use of contraceptives, douching or promiscuous sexual practices disturb the healthy microflora followed by growth of harmful aerobic or anaerobic bacteria leading to formation of grayish vaginal discharge with fishy odor [7]. Three parameters out of the following four parameters are indicative of bacterial vaginosis: presence of vaginal pH ≥ 4.7, release of fishy odor after adding 10% potassium hydroxide (KOH), presence of “clue cells” (stippled appearance of epithelial cells when covered with bacteria) and thin grey homogeneous discharge [8].

In young females, Lactobacillus spp. colonizes the vaginal environment under the influence of estrogen which produces lactic acid to set the pH to 3.8-4.5 [9]. This acidic pH is particularly important for healthy microflora. The reduced number of Lactobacilli replaced by pathogenic anaerobic and aerobic flora produces aminopeptidases to generate amines which increases the vaginal pH. Additionally, this abnormal flora produces enzymes and metabolic by-products which suppress the immune response and contribute to the severity of the infection and complications in pregnancy. Rupture of membranes, preterm delivery or postpartum sepsis have been documented as associated issues with BV. [10, 11].

Based on the demographic data of the study population, the prevalence of BV ranges from 4-64% while 12-25% females have asymptomatic BV. [12]. Both bacterial vaginosis and HCV infection lead to the development of oxidative stress which is the imbalance between generation and quenching of reactive oxygen species (ROS) [13, 14]. This leads to increased level of free radicals including O₂ and H₂O₂. ROS species have detrimental effects on the cellular integrity, also linked with the apoptosis of affected cells. These cells release antioxidants to quench ROS species which include enzymes like catalase, peroxidase, and superoxide dismutase (SOD) to convert into water and oxygen. ROS species and their respective enzymes can serve as the markers of oxidative stress [15]. This concomitant presence of oxidative markers in HCV infection and bacterial vaginosis may serve as a connection between two conditions.

The disease severity is even high when patient is encountered with secondary infections. Bacterial vaginosis is reported to be linked with the colonization and transmission of sexually transmitted viruses including HIV, HPV and HSV [15]. Although there are rare chances of venereal transmission of HCV, but strong relation of both BV and HCV positive females led to development of the study with the aim to compare the oxidative stress status in HCV-BV (coinfection) with respect to BV (mono-infection) among pregnant females.

Methods

A case control study was designed enrolling pregnant females (PF) with HCV-BF co-infection, PF with BV mono infection individuals compared with the healthy subjects i.e., PF with no infection. All the patients were selected from the gynae and obstetrics out-patient department (OPD) at Hilal-e-Ahmar Maternity Hospital, Faisalabad, Punjab
Pakistan after ethical approval (letter no. 539/HAHF/9-9-2016). Volunteer’s pregnant females (n=200) were chosen for this study and consent was taken after verbal and written explanation of the methods and risk.

These females were divided into three groups PF with HCV-BV (n=25), PF with BV (n=25) and PF control group (n=25). Inclusion criteria for PF with HCV-BV was based on following parameters (a) pregnancy (b) HCV detection (c) per vaginal examination (PV) for BV.

Pregnancy was already determined by urine pregnancy strip test beta hCG (AccuMed)®. This test uses antibodies to detect human chorionic gonadotropin (hCG). It is an ideal marker of pregnancy since it rises rapidly and consistently in early pregnancy with an accuracy of tests over 99% [16]. The pregnancy of selected individuals was further confirmed by radio-graphical imaging using ultrasound machine. Diagnosis of HCV by rapid diagnostic kit (ctK)® followed by confirmation with real time Polymerase chain reaction (RT-PCR) was done. BV status were confirmed by PV examination and Amsel criteria of bacterial vaginosis [17] was considered as the inclusion criteria for patients enrolled in the respective group.

During PV examination, vaginal swabs were collected for the microbiological study and enriched in trypticase soya broth (TSB) (Oxoid UK)® for 24 hours at 37°C. Vaginal discharge was collected with the help of cotton swab and dipped into 2 ml phosphate buffer saline (pH 7.3) (Oxoid UK)®. In falcon tube for study of oxidative stress parameter and stored at -80°C. Those females were excluded from the study which had the history of hysterectomy, AIDS, hypersensitivity to vitamin C and recent chemotherapy. Furthermore, for hematological analysis and oxidative stress analysis and HCV detection 6 ml of blood was collected from the median cephalic vein and equally divided into with and without anticoagulant vacutainer tube (BD)®.

The participants of PF with HCV & BV group were subjected for HCV detection with the aid of real-time PCR (Humacylcer). The extraction of RNA from plasma was conducted using NucleoSpin® kit. The HCV detection was carried out using Qiogong (Germany) kit i.e., based upon the amplification of single copy 5’UTR RNA sequence and measuring of the amplification product, concentration growth using reverse transcription polymerase chain reaction (RT-PCR) and fluorescence-labeled probes. HCV presence was detected by FAM fluorophore. Amplification of the internal control was visualized by HEX fluorophore.

CBC analysis was conducted with the aid of Abacus 5-part (Hungry) hematology analyzer. HB, granulocytes, a granulocytes, monocytes, leukocytes, and platelets was measured by laser light scattering technology. Oxidative stress was determined in blood plasma of all the subjects enrolled in the study.

Streaking of vaginal swab was done on Blood, MacConkey, CLED, Nutrient and MRS media (Oxoid UK)®. All were incubated at 37°C for 24 hours aerobically and anaerobically. Biochemical identification was conducted. Total bacterial count per ml was counted with the help of Breed smear method [18].

16S rRNA gene was amplified for two representative isolates, using universal primers. The amplicons were purified using QIAquick PCR purification kit Qiagen (Germany). Sequencing for molecular identification of isolates was performed using sequencing services by 1st Base (Malaysia). Sequences were submitted to GenBank data base and accession numbers were obtained.

Plasma and vaginal secretions from both group participant’s i.e., PF with HCV & BV and PF with BV along with healthy Control group were analyzed for the measurement of oxidative stress level that includes Superoxide dismutase (SOD), Catalase, Lipid peroxidation (malondialdehyde [MDA]) and Hydrogen per oxide (H2O2).

SOD activity was assessed by a colorimetric assay kit ab65354 (Abcam, Cambridge, MA, USA). The reagent utilized in the kit is a water-soluble tetrazolium salt (WST-1), which produces a water-soluble formazan dye that can be detected at 450 nm upon the reduction of WST-1 by superoxide anions. WST-1 reduction is inhibited by SOD, which catalyzes the dismutation of the superoxide anion to produce H2O2 and O2. Therefore, SOD activity was calculated based on the percent inhibition of WST-1 reduction, which in turn reflected the percent inhibition of the superoxide anions.
Catalase activity was assessed by a colorimetric assay kit ab83464 (Abcam, Cambridge, MA, USA). The catalase present in the samples reacts with hydrogen peroxide (H$_2$O$_2$) to produce water and oxygen. The unconverted H$_2$O$_2$ reacts with probe to produce a product that measured at OD 570 nm.

MDA activity was assessed by a colorimetric assay kit ab118970 (Abcam, Cambridge, MA, USA). The MDA in the samples reacts with thiobarbituric acid (TBA) to generate MDA-TBA adduct. The MDA-TBA adduct quantified at OD 532 nm. H$_2$O$_2$ activity was assessed by a colorimetric assay kit ab102500 (Abcam, Cambridge, MA, USA). The H$_2$O$_2$ in the samples react with horse radish peroxidase (HRP) reacts at OD 570 nm.

For descriptive statistics of continuous variables, means and standard deviations were calculated, whereas categorical variables were expressed as proportions. The normality of variables was evaluated by the Kolmogorov–Smirnov test. Comparisons between groups were assessed using Kruskal–Walli’s test followed by a post hoc Dunn’s Multiple Comparison Test due to small data set. Statistical significance was defined as p < 0.05. The SPSS software package version 20 and GraphPad Prism were used for all statistical analyses.

**Results**

Among the 150 participants who gave consent were included in the study, of which 75 subjects met the inclusion criteria and divided into three study groups with 25 subjects in each group. The demographic characteristics of all groups are presented in Table 1. There is a no significant difference (P > 0.05) in average age among both co-infected (PF with HCV-BV) and mono-infected (PF with BV) groups. While average weight was significantly higher among co-infected group compared to the mono-infected (P < 0.05). Moreover, the average ratio of children was found 1:2 in mono-infected and co-infected, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Total No.</th>
<th>No. of children</th>
<th>Weight in Kgs</th>
<th>Age in Years</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF With HCV-BV</td>
<td>25</td>
<td>2±1.5</td>
<td>65.15 ± 7.5</td>
<td>31 ± 3.1</td>
</tr>
<tr>
<td>PF With BV</td>
<td>25</td>
<td>1 ± 1.3</td>
<td>74.36 ± 5.9</td>
<td>30 ± 3.4</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>1 ± 1.0</td>
<td>59.08 ± 3.7</td>
<td>27 ± 2.2</td>
</tr>
</tbody>
</table>

*represents the significant difference.

Complete blood chemistry (CBC) parameters reveal that, the average granulocytes percentage (neutrophils, basophils, eosinophils, and monocytes) were observed significantly higher among the co-infected individuals (PF with HCV-BV) compared to the mono-infected (PF with BV) (P<0.05). The average lymphocytes, platelets, and blood hemoglobin level (HgB) were significantly lower among the co-infected individuals (PF with HCV-BV) compared to the mono-infected (PF with BV) (P<0.05). The results indicate the severity of disease among coinfected, compared to mono-infected group [Figure 1].
Overall, the coinfected group’s microbiological culture reports show a significantly higher frequency of pathogenic isolate i.e., *Acinetobacter* spp. Also, we observed that the Nugent score was comparatively high for the coinfected group [Table 2]. These results suggest the severity of disease in coinfected group.

Table 2: Microbiological analysis and Nugent Score.

<table>
<thead>
<tr>
<th>Nugent Score</th>
<th>Isolation Frequency (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Intermediate</td>
</tr>
<tr>
<td>(0-3)</td>
<td>(4-6)</td>
</tr>
<tr>
<td>PF with HCV-BV</td>
<td>-----</td>
</tr>
<tr>
<td>PF with BV</td>
<td>-----</td>
</tr>
<tr>
<td>Control</td>
<td>100%</td>
</tr>
</tbody>
</table>
*represents the significant difference, one representative isolate was sequenced for 16s rRNA, and accession numbers were obtained from NCBI.

Percentage inhibition of SOD by the reducing superoxide anion was significantly higher in case of PF with BV (mono-infection) compared to PF with HCV-BV (co-infection) (P<0.05). The overall trends of superoxide anion reduction were more clearly observed in vaginal secretions than the blood plasma in all the three study groups. The similar trend was observed for catalase produced [Figure 2 A and B]. These results suggest the decreasing trend of anti-oxidant levels among the coinfected group.

The MDA and H2O2 levels were significantly higher in the co-infected group compared to the mono-infected in blood plasma as well as vaginal secretions of both groups (P<0.05). The results indicate the greater oxidative stress in case of co-infection [Figure 2 C and D].

**Figure 2:** (A) Plasma and Vaginal Secretions SOD activity with a colorimetric assay kit. (B) Plasma and Vaginal Secretions catalase levels using the catalase assay kit. (C) Plasma and vaginal secretions MDA levels using the MDA assay kit. (D) Plasma and vaginal secretions H2O2 levels using the H2O2 assay kit. *Represents statistical significance. **Represents highly statistical significance.
Discussion

In recent years, much attention is given to the investigation of clinical biomarkers indicating severity of infection in pregnancy and enable early diagnosis and therapy. Pregnant females are more severely affected by the bacterial/viral infections and experience immunologic alteration as compared to non-pregnant females [19]. BV is the most common cause of abnormal vaginal discharge with a prevalence as high as 50% in non-pregnant communities and 9-23% in a population of pregnant women [20]. The main cause of BV is the disturbance in healthy microbiota, an important barrier against the pathogenic organism. The replacement of predominant lactobacillus in vagina replaced by different anaerobes is the most significant observation in case of vaginosis [21]. The study was designed with the aim to present the redox status as an important parameter for assessing the severity of the disease in HCV-BV co-infection and opting better treatment option and control for the infection.

In this study, Acinetobacter spp. significantly out-numbered the Lactobacillus spp. (P<0.05) among the HCV-BV co-infected group as compared to the mono-infected BV and the control (Table 1). A similar study from Nepal suggested that BV have a direct association with Acinetobacter spp.[22]. Acinetobacter primitively a well know, commensal bacteria that gained multidrug resistant and became pathogen is responsible for pneumonia, fever septicemia, chorioamnionitis and premature contraction during pregnancy [23, 24].

Globally, BV is a common genital problem among women seeking gynecological care. In the current study the average age of the affected participants were found to be 31 years with no significant difference (p > 0.05) among both PF with BV and HCV- BV co infected Figure 1. These findings are in accordance with the study from Nepal that report high prevalence of BV among the age group 31-40 [22]. Similarly, in India the prevalence rate of BV was found 24.4% by Nugent's method and this rate was higher among the age group 30 to 40 [25, 26].

A range of factors may explain this age and BV puzzle, such as late marriages, low fertility, demographic, and socioeconomic status of the participants. The study suggests a more detailed demographic review to be conducted to gain insights in the factors that contribute the most. According to Pakistan’s Demographic & Health (PDHS) Survey the 18.4 years is the average marriage age in Pakistan. Age factor is the major determinants of fertility in females [27]. In this study the average ratio of the children among HCV-BF Coinfected and BF-mono-infected was found to be 1:2. Late marriages is another responsible factor where unmarried females may find greater opportunities to excel their carrier, but fertility issues increase with the age. At the same time late marriages concept was not acceptable in society like Pakistan [28]. Therefore, such females having great sexual and mental stress attract towards masturbation and experience the douching and contraceptives. Studies have been reported where douching was statistically related to BV (P = 0.015) [29, 30][31, 32].

All these factors are associated with altering the normal vaginal pH and hence increase the risk factor for acquisition of sexually transmitted infection including human immunodeficiency virus (HIV) and HCV.

Another interesting fact, HCV-BV significantly correlated (P<0.05) with obesity compared to BV-mono-infected and higher Nugent score (Table 1 & 2). Similar trends of obesity and higher Nugent scores have been observed in studies reporting association of obesity with HCV and BV [33, 34]. The weight gain during pregnancy is an obvious process, apart from physiological stress and diet, hormonal imbalance such as increase in hypothalamic-pituitary-adrenal axis (HPA) activity is directly associated with weight gain during pregnancy. However, a study from Washington (USA) suggested that there is no relationship between obesity and BV [35]. This contradiction in the findings might be because of the ethnic and geographical distribution.

Globally 71 million people are living with HCV out which 7.1 million (10%) are present in Pakistan with the second largest HCV burden in the world [1, 36]. HCV causes chronic infection of the liver characterized by the persistent inflammation leading to regenerative liver fibrosis and cirrhosis [37].

Immune response against chronic HCV, initiates the production of oxidative stress [4]. Farinati et al. further reports that HCV produces more oxidative stress than other hepatitis viruses [4]. Even its core proteins NS3, NS5A, E1, E2, NS4B are directly involved in increase of oxidative stress [38, 39].
The presence of excess ROS can lead to cellular damage of DNA, lipids, and cellular protein [40]. Similarly, in the BV patients the disturbance of healthy vaginal flora, causes bulk production of ROS in the genital tract and are capable to cause vaginal epithelial cell apoptosis [13]. Some other studies have also reported gynecological diseases including fibroids, endometriosis and postoperative adhesions associated with the oxidative stress [41-43].

Noticeably, the pregnant females experience more ROS production as compared to normal females [44]. During pregnancy physiological, anatomical, and metabolic changes occur therefore, the balance in ROS is very much crucial for the developing cells of the fetus and maturation. These ROS are coming from mother body during supply of adequate nutrient and oxygen [45].

The sum of above discussion reveals that, BV, HCV, and pregnancy are directly involved in the oxidative stress in terms of ROS production. Therefore, we collectively study the oxidative stress in HCV-BV coinfected pregnant females. Antioxidants are produced as a part of physiological process of the cell to combat the ROS. They play a critical role in disease prevention by scavenging and detoxification of ROS and maintain health. Decrease in antioxidant defense system or an overproduction of free radicals results in oxidative stress which induce aging. In this study we found lower antioxidant level in vaginal secretions among coinfected then mono-infected group (figure 2A & B) indicating the severity of disease in cooinfection. These results can be related to a study where bacterial/viral coinfection (HIV-TB) had high levels of antioxidants compared to TB (mono-infection) [46]. However, in our study, the reason for lower levels of antioxidant in vaginal secretion among coinfected group is related to the ROS production. Further, we found high ROS production in blood plasma among coinfected as compared to mono-infected group (figure 2 C & D). These results are in accordance with a study where blood plasma ROS was significantly higher (P<0.05), in the (HIV-HCV) co-infected compared to the (HIV) mono-infected participants [47] although this comparative study was based on solely viral diseases. A suggested reason of high ROS production in plasma is the HCV virus however further prospective studies are required to confirm this physiological condition.

Therapies based on both antioxidants and antioxidant enzymes can be an effective approach in preventing or treating diseases. The study suggests the need for the molecular analysis of oxidative stress genes and computational approaches to find out the genetic bases and association of the disease which may provide insight knowledge to determine the exact reason.

**Conclusion**

Overall, we found the severity of disease among coinfected group. The patients from this group experienced more oxidative stress and pathogenic microbial burden. The levels of ROS and antioxidants can be used as diagnostic markers to label the disease severity, also antioxidants can be used as optimal therapeutic agents. This research could aid in the early diagnosis of hepatocellular cancer and bacterial vaginosis, as well as encourage scientists to develop an oxidative stress marker for coinfection detection.

**Limitation**

Although this study has various limitation such as the limited number of participant and demographic factors such as knowledge about douching and contraceptive use, diet and living status but the study may provide baseline knowledge about the pathophysiology of coinfection. Moreover, the study lacks the information about the therapeutic methods for the HCV and BV patients which may influence the results. Further study is required to analyze the impact of treatment among both study groups.

**Data availability**

The 16s rRNA sequence of representative isolates submitted under accession number MT269939 and MZ314512 on NCBI.
Acknowledgement

We acknowledge the role of Pathology department of Faisalabad institute of cardiology and Alkhidmat diagnostic center for providing lab facilities.

Conflict of interest

The authors declare that they have no conflict of interest.

Abbreviations

CBC Complete blood chemistry
HCV Hepatitis c Virus
BV Bacterial vaginosis
SOD Super Oxiod dismutase
MDA Malondialdehyde
ROS Reactive oxidant species
PF Pregnant females

References


