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The effect of ozone on bacterial vaginosis and how it is affected by ultrastructural changes of cells by transmission electron microscope (TEM)

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Three hundred and sixty four (364) high vagina swabs (HVS) were gathered from a hospital in the Central Region of Saudi Arabia. The following samples were identified from the following genera: *Escherichia coli, Klebsiella sp., Proteus sp., Lactobacillus sp., Micrococcus sp., Staphylococcus sp., Streptococcus sp.* as normal vaginal flora, *K. pneumoniae*, *Streptococcus* Group B and *S. aureus* as the causal agent of bacterial vaginosis. In the different age categories of women, adults had the highest percentage of infection (29.78%). The response of the isolated bacteria to antibiotics such as Amoxicillin (AMX), Amoxicillin-Clavulanic acid (AML), Cefaclor (CEC), Cefotaxime (CTX), Ceftriaxone (CRO), Cefuroxime (CXM), Cephradine (CE), Clindamycin (DA) has revealed that they have more effect on *Streptococcus* Group B, *S. aureus* and *K. pneumoniae*. Results have shown that ozone (40 µg/L) can efficiently deactivate the growth of *Streptococcus* Group B more than Ampicillin (AMP), Cotrimoxazole (SXT), Erythromycin (E), Gentamycin (GN), Ofloxacin (OFX), Peflloxacine (PEF), Tetracycline (TE), Trimethoprim (W). Also, is case of *S. aureus* with Ciprofloxacin (CIP), OFX, TE, W and *K. pneumoniae* with SXT. The state of bacterial growth and cells form of *Streptococcus* group (B), *E. coli* and *Lactobacillus* sp. by exposure to different concentrations of ozone was checked by using Transmission Electron Microscope (TEM). Bacterial cells had collapsed and shrunken patterns, were deformed, had severe rupture and destruction after being exposed to ozone; unlike in the control treatment. Results obtained through this research are considered to be the first addressing the efficiency of ozone in destroying the resistance of HVS isolated bacteria to some antibiotics, and subsequently restoring the capacity of these antibiotics in treatment again.

Key words: Bacterial vaginosis, antibacterial activity, ozone, antibiotics, *Lactobacillus*, *Streptococcus, Escherichia coli*, transmission electron microscope (TEM).

INTRODUCTION

Bacterial vaginosis affects millions of women and is associated with several serious health conditions populated by a variety of microorganisms especially bacterial infection (Steven et al., 2007). It seems that infection is not only a question of the increasing number of pathological germs, but also of decreasing number of Lactobacilli (Mercenier et al., 2002; Jahić et al., 2006; Steven et al., 2007).

The role of vaginal *Lactobacillus* as an efficient barrier against invading pathogens is of considerable interest.

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Certain Lactobacilli produce H_2O_2 and lactic acid, which normally suppress growth of anaerobes e.g. *Clostridium* species. However, in bacterial vaginosis, such virulent ones as *Bacteroides fragilis, Prevotella* species*, Escherichia coli*, *Enterococcus* sp., *Gardnerella vaginalis* and even occasional Trichomonads and fungal orga-nisms and other anaerobes proliferate and lead to decreasing of the number of Lactobacilli (Sobel, 1990; Zhou et al., 2006; Dimitonova et al., 2007; Patterson et al., 2007).

Using different antibiotics for bacterial vaginosis is very common for treatment (Eschenbach et al., 1983; Sanchez et al., 2004; Bradshaw et al., 2006). While the wide-spread use of antibiotics increases the potential for emergence of resistant strains (Traub and Leonhard, 1997; McKenna and Lams, 1998; Rouse et al., 1998). A new approach for using ozone as a treatment tool for vaginal infection was followed as it increases the action of immune system against bacteria (Jakab et al., 1995; Kachalina et al., 2000; Allen, 2002; Sunnen, 2005; Grechkanev et al., 2011).

Ozone is an activated, trivalent form of oxygen. There are two types of ozone: First is the medical (therapeutic) ozone which is generated from pure Oxygen Source and is a mixture of ozone gas and pure oxygen. The second one is Non-Medical (Industrial) ozone from Ambient Air Source (Bocci et al., 1993; Kachalina et al., 2000; Huimin et al., 2007; Maslennikov et al., 2008). Gopal et al. (1997) and Estrela et al. (2007) proved that medical ozone is a very safe and effective anti-bacterial (depending on its concentration) compared to other anti-microbial agents.

The objectives of this research are to recognize bacterial vaginosis, which exhibits resistant to antibiotics used for treatment and subsequently to evaluate the percentage of the genus that is dominant in vaginitis in relation to different categories of ages. The second part deals with different concentrations of ozone for determining its anti-bacterial effect on bacterial growth. Finally, imaging using TEM on bacterial cells before and after exposure to ozone to support its capability was examined.

MATERIALS AND METHODS

The protocol of gathering the bacterial samples

The high vaginal swab (HVS) samples were taken from the cervix of 364 female patients from 17 to 58 years, with median age of 31 years. Direct examination to detect pus cells**,** red blood cells, fungal hyphae, yeast cells and *Trichomonas* was made by examining them under a light microscope to see the type of cells present and to determine if it is normal vaginal flora or infected sample. Infected samples were cultured on different types of media: Aerobic condition O_2 incubator for inoculated Sabouraud Culture Media, facultative anaerobic condition CO_2 incubator for inoculated Blood, Chocolate Culture Media and Anaerobic condition Jar to detect *Gardnerella* were used.

Different bacterial colonies that appeared were considered normal vaginal flora in the cervix media such as *Lactobacillus* sp. which exhibited green color colonies, or other bacterial colonies like *Sterptococcus* or *Staphylococcus* colonies. In the case where *Sterptococcus* colonies appeared, Strep test was done to know which group they belong to. For *Staphylococcus*, coagulase was tested to know the type of *Staphylococcus.* If the result was positive it would be *S. aureus,* and the sensitivity to the antibiotics would be tested. When Gram-negative bacteria appeared on the culture media after 24 h and with a large number of pus cells, API test was used to determine the type of organisms and its sensitivity to the antibiotics was done. After 48 h, the previous Petri dishes as well as the other two media of Sabouraud and *Gardnerella* were checked. Normal colonies found on the Petri dishes would be recorded as "normal vaginal flora". If *Candida* were grown on the Sabouraud, the type of *Candida* was determined by taking part of the growing *Candida* and culturing it on chrom agar; if the color of the growth was green, it would be *C. albicans,* but if the color was white, it would be type of *Candida* species. Samples and isolates were collected and identified in Suliman AL-Habib medical Hospital, Riyadh, Saudi Arabia, within three months from 1st February to 30th April, 2008. Clinical data were gathered from hospitalization files.

Ozone treatment of isolated bacteria *in vitro*

Nutrient Broth suspension of 50 ml was prepared at 0.5 McFarland Standard (Adapted from Chapin and Lauderdale**,** 2003), at wavelength of 600 nm (OD_{600}) and left for 24 h at 37°C. Ozone stream was passed through a suspension of bacteria, at concentrations of 20, 40 and 60 µg/L for 30 min by using EXT120 Ozone Generator.

Bacterial colony counting before and after ozone treatment

One ml of bacterial suspension after ozone treatment was added to 9 ml of saline (1×10^{-1} dilution) in a test tube. A suitable suspension (0.01 ml) was then added to agar plates after solidification and incubated for 24 h at 37°C to be counted. Three plates for each specimen were used and the mean number of counting was taken.

Antibiotic sensitivity tests

Isolates were grown on the Muller Hinton Agar (Oxide) medium. Isolates were incubated in O_2 incubator and left for 24 h. They were then observed to determine their sensitivity and resistance to antibiotic before and after exposure to ozone, by using disk diffusion method as reported by Kaçmaz and Aksoy (2005). The effect of antibiotics such as Amoxicillin (AMX) 30 µg, Amoxicillin-Clavulanic acid (AML) 20/10µg, Ampicillin (AMP) 10 µg, Cefaclor (CEC) 30 mcg, Cefixime (CFM) 5 µg, Cefotaxime (CTX) 30 µg, Ceftriaxone (CRO) 30 µg, Cefuroxime (CXM) 30 µg, Cephradine (CE) 30 µg, Ciprofloxacin (CIP) 5 µg, Clindamycin (DA) 10 µg, Cotrimoxazole (SXT) 25 µg, Erythromycin (E) 5 µg, Gentamycin (GN) 10 µg, Levofloxacin (LEV) 5 µg, Ofloxacin (OFX) 5 µg, Peflloxacine (PEF) 5 µg, Tetracycline (TE) 10 µg, Trimethoprim (W) 2.5 µg was estimated by measuring the diameter of inhibition zone to determine the sensitivity and resistance of bacteria according to NCCLS (2002).

Transmission Electron Microscope (TEM)

To study the effect of ozone on bacterial cells by Hitachi "H-9500" 300KV TEM, Ross and Deutsch (1957)'s steps were followed. The "H-9500" is designed to support research on solid state materials and polymeric materials. The new "H-9500" utilizes modern computer control and digital cameras to enhance user-friendliness.

It is known for its ease of atomic resolution imaging, high sample throughput and a wide variety of analytical capabilities.

Statistical analysis

Data were analyzed using the statistical programs of SPSS (2001) and SAS (2008). All values are Means (± SD) obtained from triplicates. For statistical analysis, one-way ANOVA with Duncan's test was used for comparison. In all the cases, a difference was considered significant when p was ≤0.05.

RESULTS

Study of pathological predominance of bacteria according to age indicated normal vaginal flora (*E. coli, Klebsiella sp., Proteus* sp*., Lactobacillus* sp*., Micrococcus* sp*., Staphylococcus* sp*., Streptococcus* sp.) in 242 out of 364 female patients, from 17 to 58 years with median age of 31 years. The study also proved that non-pathogenic bacteria "normal flora" were predominant (4.1%) in teens (17 – 21 Y) compared to pathogenic (1.9%). HVS showed that almost one third of women (29.8%) were bacteria vaginosis positive.

In the pathogenic Gram-positive bacteria category, *Streptococcus* group B was detected in 18 cases (4.9%) other than one case of *S. aureus* (0.3%); while in the pathogenic Gram-negative bacteria case, *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* were detected in a separate case (0.3% for each). Presence of fungal elements was recorded as yeast cells, identified as *Candida* sp. in 24 cases (6.5%) and *C. albicans* in 63 cases (17.3%). Mixed infection was detected in 12 cases (3.3%) of which 9 cases were *Streptococcus* type B with *C. albicans* (2.4%), one case (0.3%) of *Streptococcus* type B with *Candida* sp. and 2 cases (0.6%) of *S. aureus* and *C. albicans.*

Interaction between bacterial isolates and ozone levels at the count of bacteria

The count of bacteria was statistically and highly significantly different (P<0.0001) (Figure 1 and Table 1). The lowest count of bacteria was observed in *K. pneumoniae* (7.5×10^8 CFU), *Klebsiella* sp. "normal flora" (2.6×10^9 CFU), *S. aureus* (7.4×10^9 CFU), *S. epidermis* (8.2×10^9 CFU), and *Streptococcus* "normal flora" (1.1×10^{10} CFU), followed by *Staphylococcus* sp. "normal flora" (2.6×10^{10} CFU) and *Lactobacillus* sp. "normal flora" (2.7×10^{10} CFU). Furthermore, the highest count of bacteria was observed in *Proteus* sp. (4.7×10^{10} CFU), *Micrococcus* sp. (4.2×10^{10} CFU), *E. coli* (3.8×10^{10} CFU), and *Streptococcus* Group B (3.7×10^{10} CFU).

Means (± SD) of count of bacterial cells by the interaction between bacterial isolates and ozone levels were highly significant (P < 0.0001), and this means that the count of bacterial cells differed significantly between the ozone levels through the species (Table 1).

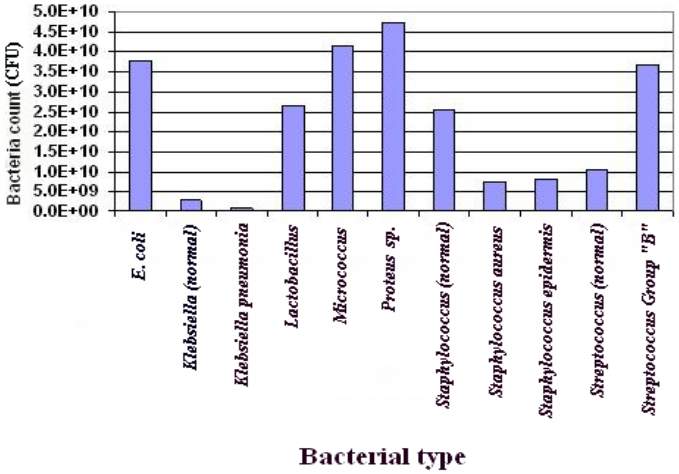

Figure 1. Total count of bacterial vaginosis isolates.

Regarding swabs, there were significant differences (P<0.0001) between them in the counts of bacteria. The 40 and 60 µg/L ozone level had statistically equal significant effect (P<0.0001) in reducing the count of total bacteria (3.3×10^7 and 1.8×10^2 CFU respectively) more than the other two levels of ozone, 0 and 20 µg/L, (4.7×10^{10} CFU each, respectively).

Effect of antibiotics on bacterial growth before and after exposure to ozone

This was concluded from Table 2 as follows: Some tested isolates of *Streptococcus* group B were resistant against the following antibiotics: AMP, SXT, E, GN, OFX, PEF, TE, and W before ozone treatment and became sensitive to these antibiotics after ozone treatment. Tested isolate of *S. aureus* became sensitive to CIP and W after ozone treatment. In another side some of the isolated swabs from *Streptococcus* group B, *S. aureus* and *K. pneumoniae* were resistant to antibiotics until after ozone exposure.

Transmission electron microscope (TEM)

The effect of ozone before and after exposure of 20, 40 and 60µg/L on the bacterial cells was studied by using TEM. *Streptococcus* Group B (327-436) before treated with ozone was normal cell in control group, cocci in chains. After exposure to ozone at 20µg/L, some components of the cells started to collapse. At the increase of ozone concentration to 40 µg/L, bacterial cells showed collapsed and shrunken patterns, deformity, cell wall destruction and cell debris. After treating with ozone concentration of 60 µg/L, cells revealed severe cell rupture and destruction (Figure 2). *E. coli* "normal vaginal flora" (326-287)

Table 1. Effect of isolates of bacterial vaginosis and ozone levels (µg/L) on the count of cells (means ± SD).

Bacteria			Ozone		
Bacterial Isolates	**Statistic**	**Total (CFU)**	**Ozone Levels**	**Statistic**	**Total (CFU)**
E. coli (normal flora)	N	84	Control	N	144
	Mean	3.8×10^{10} a		Mean	4.7×10^{10} A
	SD	6.6×10^{10}		SD	6.9×10^{10}
Klebsiella sp. (normal flora)	N	36	20 µg/L	N	144
	Mean	2.6×10^{9} c		Mean	4.7×10^{10} A
	SD	5.6×10^{9}		SD	6.9×10^{10}
Klebsiella pneumoniae	N	12	40 µg/L	N	144
	Mean	7.5×10^{8} c		Mean	3.3×10^{7} B
	SD	8.3×10^{8}		SD	2.1×10^{8}
Lactobacillus sp. (normal flora)	N	48	60 µg/L	N	144
	Mean	2.7×10^{10}ab		Mean	1.8×10^{2} B
	SD	4.4×10^{10}		SD	1.2×10^{3}
Micrococcus sp. (normal flora)	N	36			
	Mean	4.2×10^{10} a			
	SD	5.8×10^{10}			
Proteus sp.(normal flora)	N	24			
	Mean	4.7×10^{10} a			
	SD	4.8×10^{10}			
Staphylococcus sp. (normal flora)	N	36			
	Mean	2.6×10^{10}ab			
	SD	5.5×10^{10}			
Staphylococcus aureus	N	36			
	Mean	7.4×10^{9}bc			
	SD	1.4×10^{10}			
Staphylococcus epidermis	N	12			
	Mean	8.2×10^{9} bc			
	SD	1.1×10^{10}			
Streptococcus sp. (normal flora)	N	156			
	Mean	1.1×10^{10}bc			
	SD	4.9×10^{10}			
Streptococcus Group "B"	N	96			
	Mean	3.7×10^{10} a			
	SD	7.1×10^{10}			

Analysis of variance

SOV	df	Mean Squares (CFU)	F Value	Sig.
Bacteria (B)	10	1.2×10^{22}	8.1	0.000****
Swabs/ (B)	37	1.1×10^{22}	7.7	0.000****
Ozone (O)	3	1.1×10^{23}	72.8	0.000****
B x O	30	4.0×10^{21}	2.7	0.000****
Error	495	1.5×10^{21}		

****P < 0.0001; Means with no common superscript (capital or small) are significantly different (P < 0.05).

had normal short rods and single cells in control group. After exposure to 20 µg/L of ozone, cell components started to collapse. Respectively, cells showed collapsed and shrunken patterns of cell deformity, cell wall destruction and cell debris after exposure to 40 µg/L of ozone. After exposure to 60 µg/L of ozone, bacteria showed severe cell rupture and destruction (Figure 3).

Lactobacillus sp. "normal vaginal flora" (428-160) had

Table 2. Effect of antibiotics on isolates of bacterial vaginosis growth after exposure to ozone at concentrations 40 µg/L.

Isolate	*Streptococcus* Group B							*S. aureus*	*K. pneumoniae*
Antibiotic	**320- 159**	**331- 179**	**324-444**	**327-234**	**424-268**	**427-436**	**427-442**	**326-174**	**327-445**
Amoxicillin (AMX) 30 µg	S*	S	S	S	S	S	S	S	S
	S**	S	S	S	S	S	S	S	S
Amoxicillin-Clavulanic acid (AML) 20/10µg	S	S	S	S	S	S	S	S	S
	S	S	S	S	S	S	S	S	S
Ampicillin (AMP) 10 µg	S	S	S	**R**	S	S	S	S	**R**
	S	S	S	**S**	S	S	S	S	**R**
Cefaclor (CEC) 30 mcg	S	S	S	S	S	S	S	NT***	S
	S	S	S	S	S	S	S		S
Cefixime (CFM) 5 µg	S	S	S	S	S	S	S	**R**	S
	S	S	S	S	S	S	S	**R**	S
Cefotaxime (CTX) 30 µg	S	S	S	S	S	S	S	S	S
	S	S	S	S	S	S	S	S	S
Ceftriaxone (CRO) 30 µg	S	S	S	S	S	S		S	S
	S	S	S	S	S	S	NT	S	S
Cefuroxime (CXM) 30 µg	S	S	S	S	S	S		S	S
	S	S	S	S	S	S	NT	S	S
Cephradine (CE) 30 µg	S	S	S	S	S	S	S	S	S
	S	S	S	S	S	S	S	S	S
Ciprofloxacin (CIP) 5 µg		S	S	S	S	S		**R**	S
	NT	S	S	S	S	S	NT	**S**	S
Clindamycin (DA) 10 µg	S	S	S	S	S	S	S		S
	S	S	S	S	S	S	S	NT	S
Cotrimoxazole (SXT) 25 µg	**R**	**R**	**R**	**R**	**R**	S	**R**	S	**I**
	S	**I**	**S**	**S**	**R**	S	**S**	S	**S**
Erythromycin (E) 5 µg	NT	**R**	**R**	S	S	S	S	**R**	NT
		R	**S**	S	S	S	S	**R**	
Gentamycin (GN) 10 µg	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S
	S	**S**	**S**	**S**	**I**	**S**	**S**	S	S
Levofloxacin 1 (LEV) 15 µg	S	S	S	S	S	S	S	**R**	S
	S	S	S	S	S	S	S	**R**	S
Ofloxacin (OFX) 5 µg	**R**	S	S	S	S	S	S	**R**	S
	S	S	S	S	S	S	S	**I**	S
Pefloxacine (PEF) 5 µg	**R**	**R**	**R**	**R**	NT	NT	**R**	NT	NT
	S	**I**	**I**	**R**			**I**		
Tetracycline (TE) 10 µg	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	S
	I	**I**	**S**	S	**R**	**R**	**R**	**I**	S
Trimethoprim (W) 2.5 µg	NT	NT	NT	NT	NT	NT	**R**	**R**	NT
							S	**S**	

R: Resistance, I: Intermediate, S: Sensitive *before exposure to ozone, **after exposure to ozone, ***NT = Not Tested.

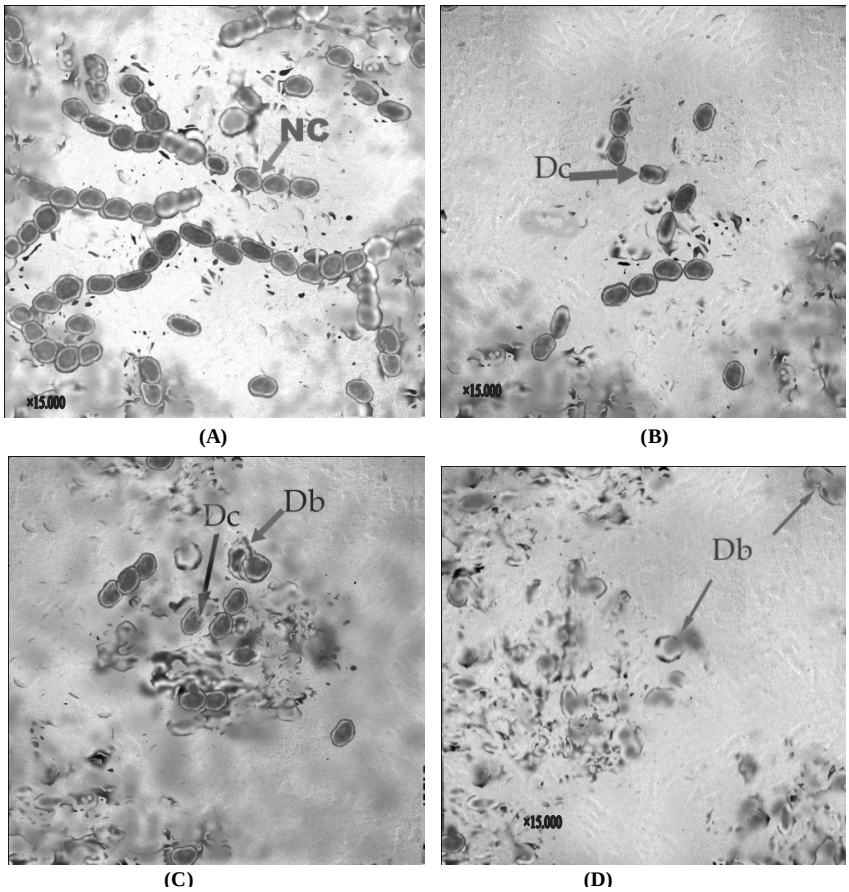

Figure 2. Transmission electron microscope of *Streptococcus* Group B, the causal agent of bacterial vaginosis, (327-436) treated with ozone. (A): Normal cell (Nc) of control group, Cocci in Chains. (B): Bacteria treated with ozone for 20 µg/L, showed start of collapsed of cell component in some cells. (C): Bacteria treated with ozone 40 µg/L, respectively, showed collapsed and shrunken patterns of cell deformity (Dc), cell wall destruction and cell debris (Db). (D): Bacteria treated with ozone for 60 µg/L, showed severe cell rupture, destruction and cell debris (Db). Magnification 1,500 x.

normal long rods and single cells in control group. After exposure to 20 µg/L of ozone, components in some cells started to collapse. After exposure to 40 µg/L of ozone, bacteria showed collapsed and shrunken patterns of cell deformity, cell wall destruction and cell debris. There was severe cell rupture and destruction, after exposure to 60 µg/L of ozone (Figure 4).

DISCUSSION

Results indicated that adults were most vulnerable to bacterial vaginosis; this can be due to menstrual cycle (Fair, 1970; Syed and Braverman, 2004), which causes changes in vaginal pH (Hemalatha et al., 2013). As a result, pathogenic bacteria were embedded in vaginal epithelial cells shed(Stameyetal.,1978;Svanborg-EdénandSvennerholm, 1978).

The count of tested bacteria differed significantly between the ozone levels through the isolates of bacteria. Similar results showed the bactericidal effects of ozone and have been documented on a wide variety of organisms, including Gram-positive bacteria: *Streptococcus* (Baysan et al., 2000) and S. aureus (Kowalski et al., 1998; Velano et al., 2001; effect of ozone appeared to inhibit growth and caused the death of Gram-negative tested bacteria: *E. coli* (Kowalski et al., 1998; Thanomsub et al., 2002). Results in great spectrum of antibiotics sensitive to bacterial vaginosis infection with different degrees from resistance, intermediate and sensitivity were exhibited in tested isolates. Similar results were obtained by Simoes et al. (2004). One out of 7 tested isolates of *Streptococcus* group B was sensitive to SXT. These results are close to the results given by Traub and Leonhard (1997) who indicated that all *Streptococcus* group B isolates were resistant to SXT.

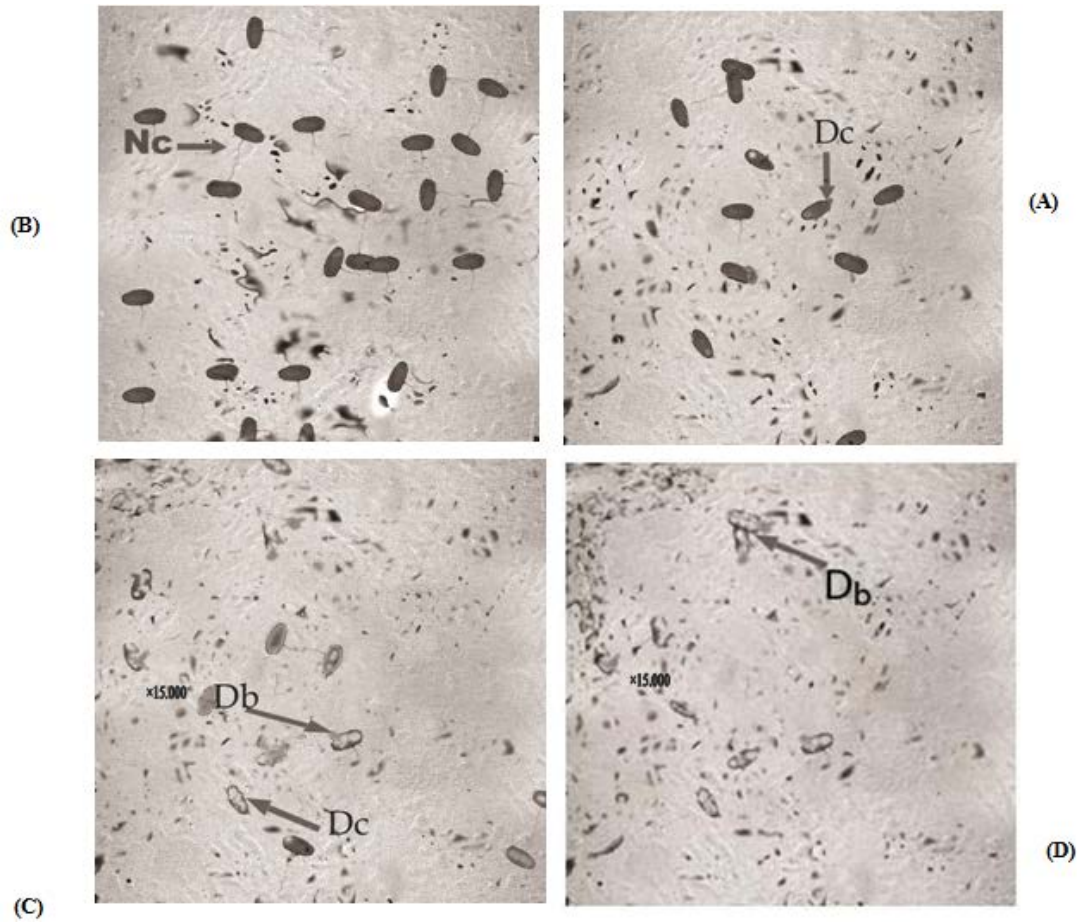

Figure 3. Transmission electron microscope of *E. coli* normal vaginal flora (326-287) treated with ozone. (A): Normal cell (Nc) of control group single cell, short rod-shaped. (B): Bacteria treated with ozone at 20 µg/L, showed start of collapsed of cell component in some cells. (C): Bacteria treated with ozone 40 µg/L, respectively, showed collapsed and shrunken patterns of cell deformity (Dc), cell wall destruction and cell debris (Db). (D): Bacteria treated with ozone at 60 µg/L showed severe cell rupture, destruction and cell debris (Db). Magnification 1,500 x.

All tested isolates of *Streptococcus* group B were modified to sensitivity towards GN after ozone treatment. In another manner, some *Streptococcus* group B isolates, *S. aureus* and *K. pneumoniae* were either intermediate or resistant against definite antibiotics before treatment, and became sensitive to the same antibiotics after exposure to ozone.

The loss of antibiotic resistance in the present results can be considered a positive trait for using ozone therapy or combined with antibiotic; this treatment failed as a result of its resistance against bacterial vaginosis. Interestingly, none of sensitive isolates were mutated to antibiotic(s) resistant after exposure to the tested ozone level.

In the effect of the count of bacteria by ozone exposure, results have shown that 40 µg/L for 30 min is the optimal level to reach the required efficiency of ozone *in vitro*, whereas less or more than that have no or the same significant effect respectively.

To detect the effect of ozone on normal vaginal flora and pathogenic bacteria, Electron Microscope imaging was done by using TEM. After exposure to ozone of 20-60µg/L for 30 min, bacterial cell appeared intact and normal in control group, ultra structure changed, morphology surface was damaged and there was collapsed and shrunken pattern of cell deformity; but when bacteria were treated with ozone of 60 µg/L, there was severe cell rupture and destruction.

The mechanism of ozone action on bacterial cells disrupts the integrity of bacterial cell enveloped through oxidation of the phospholipids and lipoproteins (Jani et al., 2012). The cell envelope of Gram-negative bacteria such as *E. coli* is a complex multilayer system composed of an inner cytoplasmic membrane made of phospholipids and proteins invigilating into the cytoplasm, a peptide-glycan layer, and an outer membrane of polymers such as polysaccharides. Gram-positive cells have a less complex,

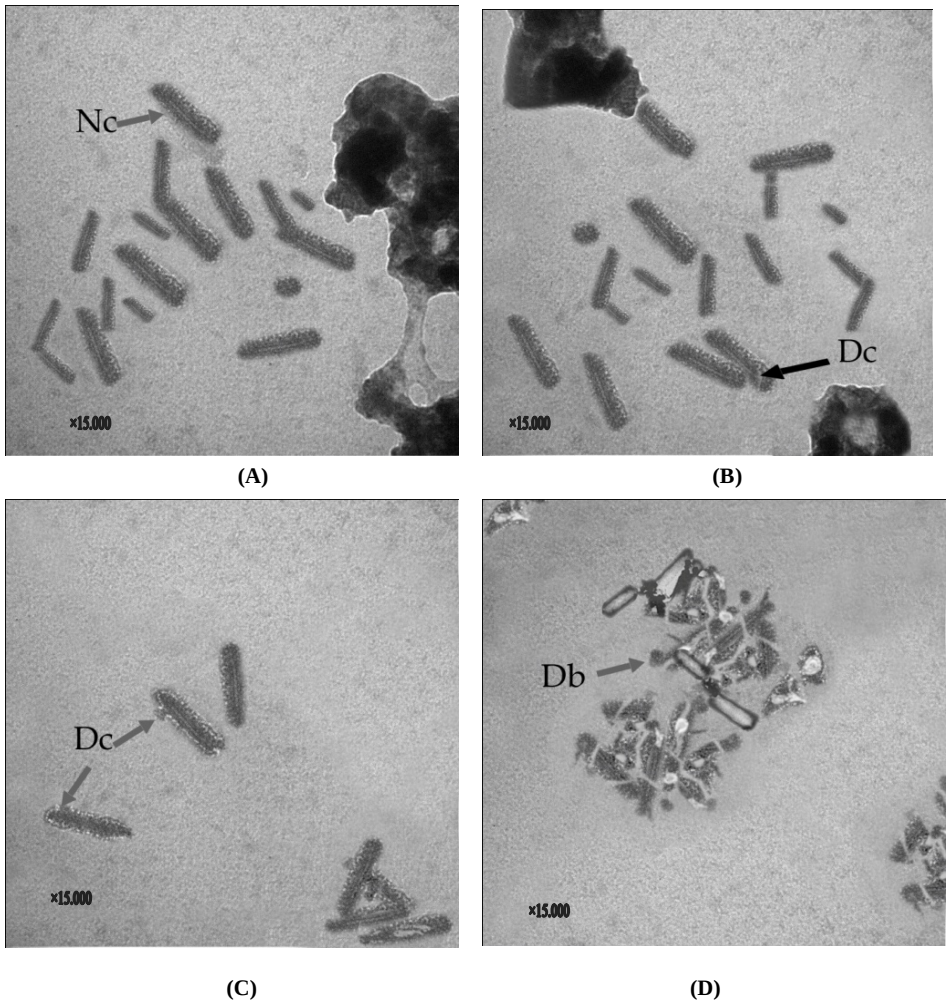

Figure 4. Transmission electron microscope of *Lactobacillus* sp. normal vaginal flora (428-160) treated with ozone. (A): Normal cell (Nc) of control group single cell, long rod-shaped. (B): Bacteria treated with ozone at 20 µg/L, showed start of collapsed of cell component in some cells. (C): Bacteria treated with ozone at 40 µg/L, respectively, showed collapsed and shrunken patterns of cell deformity (Dc). (D): Bacteria treated with ozone at 60 µg/L showed severe cell rupture, destruction and cell debris (Db). Magnification 1,500 x.

three layer envelope with a thick peptidoglycan middle layer. The most cited explanation for ozone's bactericidal effects centers on disruption of envelope integrity through peroxidation of phospholipids and lipoproteins. There is evidence of interaction with proteins as well (Mudd et al., 1969).

The importance of lactobacilli was proved by Juárez et al. (2011), who showed inhibitory activity of lactobacilli against urogenital pathogens. Thus, the most obvious finding to emerge from this study is that the main weakness of ozone is that it has the same deadly impact on normal flora especially vaginal *Lactobacillus* isolates, as well as bacterial vaginosis.

The researchers believed that ozone may be used as an alternative therapy for treatment of various diseases, although it has dangerous effects (Mandhare et al., 2012). On the other hand, Boyko and Kulida (2013) presented the results of testing the effectiveness of medical ozone in preparing for pregnancy of women with bacterial vaginosis and miscarriage of early history. Regarding Vulvovaginal candidiasis, which remains one of the most important problems in Obstetrics and Gynecology, Kotova at al. (2013) found that the result of combined use of ozone therapy and Tomed-Aqua (vaginal instillation) served as the rationale for their use in the therapy of recurrent vulvovaginal candidiasis.

Thus, the results obtained through this research have strengthened the direction of the use of ozone, particularly its efficiency on loss of resistance of HVS isolated bacteria towards some antibiotics and restoring the capa-

city of these antibiotics in treatment again, as well as how it affects ultrastructural changes of cells up till severe cell rupture and destruction.

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