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Original Research Article

OZONE APPLICATION FOR PREVENTING FUNGAL INFECTION IN DIABETIC FOOT ULCERS

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SUMMARY

Diabetes mellitus is a chronic disorder that affects a large segment of the human population and is a major public health problem. In diabetic patients, mycotic infections may increase the risk of developing diabetic foot syndrome. A total of 60 type 2 diabetic patients with non-healing diabetic foot ulcers (DFU) were recruited for the study. Among them, 72% (43/60) had yeast and mold infections. The pathogenic yeasts were noted in 60% of the patients, of which Candida (C.) species predominated. Molds were isolated from 40% of the infected patients, of which Aspergillus (A.) species preponderated (24%). The association of fungal spores with DFU was recorded with scanning electron microscope (SEM). Assessment of ozone for preventing fungal infection of DFU was performed by recording the inhibition efficacy (IE %) of gaseous ozone. Spore viability of C. albicans was reduced by over 99.5% at 3 ppm ozone concentration after 180 min' exposure time. Prevention of mycelial growth in A. flavus was detected with 100% IE at 3 ppm ozone after 210 min. With increasing duration of ozone exposure, the membrane permeability of A. flavus was compromised as detected by protein and nucleic acid leakages accompanied with lipid and tryptophan oxidation. The study also determined the efficiency of ozonation in degrading mycotoxins produced by most dominant mycotoxigenic fungal species. The production of aflatoxins and trichothecene toxins was greatly inhibited at 3 ppm ozone after 180 min. The efficacy of ozone-like supportive therapeutic treatment of patients with DFU was compared with target antimycotic therapy and was performed only by measuring the area of lesions.

INTRODUCTION

Diabetes is common with over 194 million people having the condition worldwide (1). With an estimated 50 million people worldwide, all populations and age groups are affected by diabetes mellitus (DM) (2). The

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annual incidence of new cases of type 1 and type 2 DM in the USA is estimated to be 30 000 and 625 000, respectively (3). The prevalence of DM was estimated to be 2.8% in 2000 and projected to 4.4% in the year 2030, with a total number of people with diabetes expected to rise from 171 million in 2000 to 366 million in 2030 (4). Patients with diabetes represent a unique group of individuals who appear more prone to develop infections than others. Several mechanisms have been proposed to explain the association between diabetes and infections. However, few conclusive studies exist and a considerable debate is going on regarding the evidence for this predisposition.

Foot ulcers are a much feared complication of diabetes, with recent studies suggesting that lifetime risk of developing a foot ulcer in diabetic patients may be as high as 25% (5). Diabetic foot infections are one of the major causes of morbidity and mortality, especially in developing countries (6,7) due to illiteracy, poor socioeconomic status, bare-foot walking and inadequate facilities for diabetes care. In Germany alone, approximately 22,000 amputations are performed in people with diabetic foot ulcer (DFU) every year (8). Infection is most often a consequence of foot ulceration, which typically follows trauma to a neuropathic foot (9). Foot infections are now the most common proximate and non-traumatic cause of leg amputation (10,11). It has been estimated that the risk of leg amputation is 15-46 times higher in diabetics than in nondiabetics. In addition, foot complications now account for the most frequent reason for hospitalization in diabetic patients (12). Patients with DFU can also be at a higher risk of death due to related cardiovascular disease (13).

The most common organisms involved in diabetic foot infections are *Pseudomonas (P.) aeruginosa, Staphylococcus (S.) aureus, Escherichia (E.) coli,* and *Staphylococcus (S.) epidermidis* (12). There have been some reports of an increased incidence of fungal infections (dermatophytosis and candidiasis) of interdigital spaces and nails in the toes of diabetic patients, as well as of the association of these infections with the development of severe and deep inflammatory processes in feet (2,14). Mixed bacterial and fungal infections have been reported in 21.4% and

fungal infections alone in 5.8% of patients with diabetic foot wounds (15). Poly-microbial infection is found to be the single largest precursor for approximately 85% of the amputations among patients with diabetic foot wounds (16,17). In spite of this evidence for mixed bacterial and fungal infections in diabetic foot wounds, current clinical practice is targeted only against bacterial infections, but not against fungal infections.

Ozone therapy, or more specifically, the main ozonated autohemotherapy (O_3-AHT), has been used for almost 40 years. The first report on ozone therapy was published by Wolff in 1974 (18). In recent years, emphasis and attention have been focused on the use of medical ozone (19). Repeated administration of ozone in non-toxic doses might play a role in the control of diabetes and its complications (20). Ozone has been used for many years as a method ancillary to the usual treatment for foot ulcers in people with diabetes (21), especially in those cases in which traditional treatment methods have not proven satisfactory. Any treatment capable of stabilizing oxygen metabolism and modulating oxidative stress, accompanied by germicidal actions, can improve the quality of life of diabetic patients. Ozone can exert protective effects by oxidative preconditioning, stimulating and/or preserving the endogenous antioxidant systems, and by blocking the xanthine/xanthine oxidase pathway for reactive oxygen species (ROS) generation. Ozone has been used with good results in the treatment of patients with diabetic foot because of its germicidal properties and its influence on the processes of oxygen metabolism, and other effects (22).

Ozone is a strong, fast and broad-spectrum antimicrobial agent that works effectively against bacteria, bacterial spores, viruses, fungi, fungal spores and protozoa (23). Ozone is a powerful oxidizing agent, which finds mounting applications in many different fields to kill microbes (24). Ozone has inhibitory effect on a variety of microorganisms (25). Ozonated water has a rapid antimicrobial effect on microorganisms (26).

The presence of ROS generated by ozone leads to the induction of lipid peroxidation, therefore affecting the structure of the membranes (27-30). Ozone kills spores and viruses as it oxidizes DNA in the spore as well as in virus (23). Ozone reacts with proteins and causes oxidation of polypeptide backbone of the protein, peptide bond cleavage, protein-protein cross-linking, and a range of amino acid side chain modifications (31). Although all amino acids are potential targets for oxidation by ozone, the major aromatic amino acids tyrosine, tryptophan, phenylalanine, sulfur containing amino acids cysteine, methionine as well as the aliphatic amino acids arginine, lysine, proline and histidine appear especially sensitive to oxidation (32).

Mycotoxins are highly toxic, mutagenic and carcinogenic compounds produced by fungi (33). More recently, ozone gas was demonstrated as being effective in chemically modifying a variety of non-trichothecene mycotoxins (aflatoxins B_1, B_2, G_1, and G_2, cyclopiazonic acid, fumonisin B_1, ochratoxin A, patulin, secalonic acid, and zearalenone) and reducing their biological activity in the performed bioassays (34,35). Saturated aqueous ozone (25 ppm) degraded trichothecene mycotoxins to materials that were not detected by UV or MS, while at lower level of ozone (0.25 PPM) intermediate products were observed (36). Although several studies have been undertaken to evaluate the effects of ozone gas in reducing mycotoxin levels in agricultural products, none of them deals with those produced by fungi isolated from DFU.

The risk of infections is higher in patients with diabetes compared with controls. As individuals with diabetes age, the risk of microvascular and macrovascular complications increases several fold; these complications presumably increase the risk of infection further (37,38). Defects in the function of lymphocytes, neutrophils and monocytes contribute to the impact of infectious diseases on individuals with diabetes. Polymorphonuclear neutrophils (PMNs) in these patients show alterations in chemotaxis, adherence, phagocytosis, intracellular killing, and bactericidal activity, accompanied by decreased levels of leukotriene B4, prostaglandin E, and thromboxane

B2 (39,40). Although spontaneous activation of PMNs with increased free-radical activation has been observed, neutrophil response after stimulation of free radicals was lower in patients with diabetes. Some experts believe that hyperglycemia leads to low-level persistent activation of PMNs, resulting in a tolerant state with a less exuberant response to infection (41). Monocytes in patients with diabetes have decreased levels of phagocytosis; decreased lymphocyte function in these patients has also been described. Conversely, there is evidence that improving glycemic control improves cellular immunity (42).

Therefore, the objectives of this study were to evaluate the incidence of pathogenic fungi on deep ulcerated tissue samples detected by microbiologic and histopathologic methods where fungal contaminated foot samples were examined by scanning electron microscopy (SEM), and to investigate the efficacy of gaseous ozone on the inactivation of fungi associated with diabetic foot tissue. At the same time, this study aimed to apply ozone to the ulcer colonized by fungi, thus preventing the development of infection. The effect of ozone exposure on lipid peroxidation, tryptophan oxidation, protein, and nucleic acid leakage was recorded. The role of ozone in degrading mycotoxins produced by the most prevalent mycotoxigenic fungal species was also examined.

MATERIALS AND METHODS

Isolation and identification of fungal species from diabetic foot ulcers

The study was carried out over a period of 4 months, from January 2012 to April 2012. Sixty people with diabetes and foot ulcer hospitalized in National Diabetes and Endocrinology Institute, Cairo University, Egypt, were eligible to participate in the study. The study was approved by the scientific and ethics committees of the institution. All patients gave their informed consent to being enrolled after

receiving adequate information on the study. Pregnant women or nursing mothers were excluded from the study.

Tissue specimens were obtained from the depth of the wound of the ulcer (taking aseptic precautions) using a sterile blade/knife after debridement. To avoid isolation of colonizing flora, the wound was first thoroughly cleaned with normal saline. Samples were transferred to the laboratory within an hour in sterile containers and transported in a sterile solution of normal saline. The necrotic areas of the tissues were mounted on KOH and tissue samples (1 g) were inoculated to Czapek-Dox agar (CDA) and Sabouraud dextrose agar (SDA) (Sigma, Germany). The samples were incubated both at room temperature and 37 °C for one month and evaluated daily for growth of fungal cultures. Yeast-like growth on SDA was evaluated for germ tube formation, urease production, sugar fermentation, assimilation of sugars, and microscopic and macroscopic appearance (43,44). Fungal cultures were identified by microscopic and macroscopic appearance according to various manuals (45-49).

Ozone treatment

Ozone was generated *via* a controlled flow of oxygen through a corona discharge in the ozone generator (Ozomaxe, Egypt, ozo-3vtt). The ozone was fed into both chambers where the ozone measurement and ozone treatment were done. Ozone measurement was done by an ozone analyzer (Inusa, H1, ver. 5.73) with a detection limit of 1.0 ppb. Four ozone concentrations of 1, 2, 3 and 4 ppm were tested under different exposure times: 0 (control), 30, 60, 90, 120, 150, 180 and 210 minutes (50).

Electron microscopy

The samples were coated by gold sputter coat (SPI-Module) and examined by scanning SEM (JEOL-JSM-5500 LV) by using high vacuum mode at the Regional Center of Mycology and Biotechnology, Cairo, Egypt to study the fungal deteriorated DFU.

Preparation of fungal spores and evaluation of spore germination

Spores from most dominant yeasts were dispersed onto glass microscope cover slips (22×22 mm) by inverting and repetitively tapping a culture dish over a cardboard cylinder (70.5 cm tall × 18 cm in diameter) and allowing the conidia to settle on cover slips for 1 min. Pairs of cover slips were transferred to single Petri dish. The Petri dishes were left uncovered. Petri dishes were removed after precise exposure times. Cover slips were then inverted onto the SDA media in a Petri dish. The cover slip was removed and 30 μL of sterile distilled water added to the spores and spread across an area twice the size of a cover slip. The conidia were incubated at 20 ℃ for 24 h. After incubation, the spores were fixed with 2 mL of 70% ethanol for 2 min and then 200 conidia *per* Petri dish were selected randomly under a light microscope and examined for germination incidence. The conidia were counted as germinated if the germ-tube length was greater than the diameter of the spore. For each experiment, the germination incidence of non treated spores at time zero was also assessed as the absolute control. The germination incidence was determined by counting the number of spores germinated out of 200 randomly chosen spores (51).

Preparation of fungal mycelia and evaluation of their growth

The selected dominant fungal species were subcultured on CDA media and collected when 7 days old. The 10-mm agar discs (diameter) were obtained using a cork borer. The mycelial agar discs were centered on CDA media for ozone treatment. A set of Petri plates were left uncovered inside the ozone chamber. Following treatment, the Petri plates with agar discs were placed in the incubator for 7 days. The X and Y axes of the mycelial growth were measured in millimeters. An average of the X and Y axes was used to obtain the average diameter of mycelia growth (51). Inhibition efficiency (IE %) was calculated using the following formula: IE= $r_0 - r_1/ r_0 \times 100$, where r_0 and r_1 are radial growth in the absence and presence of ozone, respectively.

Determination of lipid oxidation

In this experiment, the mycelial growth of test fungal species was exposed to ozone as previously described. Then, mycelial discs (10 mm in diameter) were inoculated in conical flasks containing 100 mL of Czapek-Dox media. Five flasks were used for each treatment and control. All flasks were incubated on a rotary shaker at 150 rpm at 28 °C for 10 days. Filtrates were collected for malondialdehyde determination by thiobarbituric fluorometric assay at 553 nm according to the method described by Komanapalli and Lau (52).

Protein determination

Protein concentration in the ozonated selected dominant fungal species was determined using Coomassie protein assay reagent (Sigma-Aldrich Chemie GmbH). The absorbance of the protein/dye mixture was measured spectrophotometrically at 595 nm. The amount of protein was calculated according to the method described by Komanapalli and Lau (52).

Determination of nucleic acid leakage

Determination of DNA was carried out quantitatively according to the method described by Burton (53) by measuring the color developed after treating the extracted DNA with diphenylamine reagent and the absorbance was measured at 600 nm. The colorimetric analysis of ribose sugar using orcinol reaction (54) was applied for quantitative determination of RNA.

Mycotoxin production

In this experiment, two mycotoxigenic fungi were selected, namely, *Aspergillus (A.) flavus* and *Fusarium (F.) oxysporum*. These fungi were exposed to ozone gas at a concentration of 3 ppm for 0, 30, 60 90, 120 150, and 210 min, as mentioned above.

Aflatoxin determination

Aflatoxin determination in samples was carried out using the AOAC approved multifunctional column method (55). Five g of biomass of tested culture, which grew on CDA media, was combined with 10 mL acetonitrile-water (9:1) and blended for 2 min at high speed. The extract was filtered under vacuum (56). Analysis of aflatoxins was performed on a model *HP*1050 HPLC equipped with UV detector. Separations and determinations were performed on RP18 (ODS) column (length 150 mm). The mobile phase was methanol:acetic acid:water (20:20:60 v/v/v) and wavelength was 365 nm, while flow rate was 1 mL/min (57).

Determination of trichothecene toxins

Erlenmeyer flasks containing 250 mL of Czapek-Dox medium supplemented with 10 g of peptone *per* liter were inoculated with spores of *Fusarium oxysporum* and incubated at 27 °C for 5 days on a rotary shaker operating at 150 rpm. Inoculum for jar fermentor was produced according to the method described by Ueno *et al*. (58). Cultures were filtered; the crude toxin was prepared from culture filtrate according to the method previously reported by Ueno *et al*. (59), and trichothecenes in the crude toxin were assayed by HPLC using trichothecene standards prepared according to the method described by Jiménez *et al*. (60).

Therapeutic efficacy of ozone in patients with diabetic foot

Patients were randomized to three different groups of treatment: 1) antifungal therapy; 20 patients received fluconazole 150 mg/day for 20 days (15); 2) ozone; 20 patients were treated daily with ozone (generated by an OZOMED equipment, National Diabetes and Endocrinology Institute, Egypt), 20 sessions, by rectal insufflation (with an ozone dose of 10 mg, ozone concentration: 50 mg/L) and locally. For local ozone treatment, the lesion was covered with a plastic bag, sealed to the leg, which was then put under vacuum in order to eliminate the air inside it. Afterwards, the bag was refilled with ozone at a concentration of 60 mg/L. The patient remained with the plastic bag for 1 h. After that, the bag was removed and the lesion was covered with ozonized olive oil (Oleozon, Ozomaxe company, Egypt) (61); and 3) control group; 20 patients neither treated with fluconazole nor ozone but subjected to

standard care only. A trained podiatrist measured the wound size before and after each treatment. In order to measure the complete dimension of the diabetic foot wounds, a sterile, thin, polythene sheet was placed over the wound base and another sterile polythene sheet was wrapped over that and wound margins were traced with a marker. The maximum length and breadth (perpendicular to each other) were measured to calculate the wound surface area (WSA). All patients were followed up every 2 weeks for 14 weeks or until they met the treatment outcomes (15).

Statistical analysis

The Statistical Program for Scientific Studies package (SPSS 12.0 for Windows, SPSS Inc.) was used to perform statistical analyses. Data were presented as means with standard deviations (mean± SD). Statistical significance was determined by Duncan test and one-way ANOVA. A p value of less than 0.05 was considered statistically significant.

RESULTS

### Isolation and identification of fungal species

Among the 60 patients with diabetic foot infections, 42 (60%) were males and 18 (40%) females. The age range of the study population was between 48 and 69 years. The mean duration of diabetes was 11.3 (range 2-20) years. The prevalence of mold and yeast isolations was 72% of the 60 patients studied. The fungal species isolated were *Candida* spp., *Trichosporon (T.)* spp., *Aspergillus (A.)* spp., *Fusarium (F.)* spp. and *Penicillium (P.)* spp. Fungal pathogens were not detected in 17 patients. Along with fungal cultures identified, 60% were yeast isolates and 40% molds. *Candida* was the major isolated species (72%). Among the isolates, 44% (96/215) were *C. albicans*, 14.4% (31/215) *C. parapsilosis*, 8.3% (18/215) *C. tropicali*, and 4.6% (10/215) C. *krusei*. *Trichosporon cutaneum* and *T. capitatum* represented 2% and 1%, respectively (Table 1). The data also revealed that concerning filamentous fungi, *Aspergillus* was the leading genus (24.1%); *A. flavus* (20.9%) and *A.*

fumigatus (3.2%) were detected. The other mold species isolated were *F. oxysporum* (4%) and *P. chrysogenum* (1%).

Scanning electron microscope (SEM) of fungal infected diabetic foot ulcer

The observation of DFU documented extensive yeast conidia and fungal hyphae in association with infection (Fig. 1).

Figure 1. **SEM micrograph of yeast conidia and mold hypha associated with diabetic foot ulcer sample; scale bar = 22 μm**
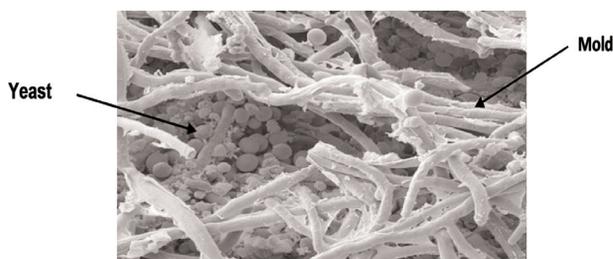
The effect of ozone treatment on spore viability of C. albicans

C. albicans spores were treated with 1, 2, 3, and 4 ppm of ozone for 0 (control), 30, 60, 90, 120, 150, and 210 minutes. Overall, the effectiveness of ozone treatments on spore germination was a function of ozone concentration and treatment duration. The results presented in Figure 2 revealed that the gradual increase in ozone concentration induced progressive retardation of the spore viability of *C. albicans*. The inhibition was more pronounced with the extension of exposure time. There was complete inhibition of the percentage of spore viability and germ tube formation in *C. albicans* when exposed to 3 ppm for 180 min (Fig. 3). All spores failed to germinate at 4 ppm at all exposure times.

The effect of ozone treatment on mycelial growth of A. flavus

The diameter of mycelial growth of *A. flavus* was determined. Table 2 shows that any increase in the exposure time at any concentration of gaseous ozone

Table 1. **Spectrum of yeast and filamentous fungi isolated from diabetic foot ulcer**

Sample no.	Species	Frequency (no. of isolates)	Percentage
1	*Candida albicans*	96	44
2	*Aspergillus flavus*	45	20.9
3	*Candida parapsilosis*	31	14.4
4	*Candida tropicalis*	18	8.3
5	*Candida krusei*	10	4.6
6	*Aspergillus fumigatus*	7	3.2
7	*Fusarium oxysporum*	4	1.8
8	*Trichosporon cutaneum*	2	0.9
9	*Trichosporon capitatum*	1	0.4
10	*Penicillium chrysogenum*	1	0.4
Total		215	100

Table 2. **Effect of different concentrations of gaseous ozone on radial growth (RG) (mm) of *A. flavus* and inhibition efficiency (IE %) at different exposure times**

Fungal species			*A. flavus*			
Ozone concn. (ppm)			1	2	3	4
	0	RG	$_a90^a$	$_a90^a$	$_a90^a$	$_a90^a$
		IE%	0	0	0	0
	30	RG	$_a80^b$	$_b72^b$	$_c69^b$	$_d0^a$
		IE%	11.11	20	23.33	100
	60	RG	$_a69^c$	$_b65^c$	$_c58^c$	$_d0^a$
		IE%	23.33	27.77	35.5	100
	90	RG	$_a59^d$	$_b55^d$	$_c48^d$	$_d0^a$
		IE%	34.44	38.88	46.66	100
Exposure time (min)	120	RG	$_a51^e$	$_b47^e$	$_c40^e$	$_d0^a$
		IE%	43.33	47.77	55.55	100
	150	RG	$_a40^f$	$_b37^f$	$_c29^f$	$_d0^a$
		IE%	55.55	58.88	67.77	100
	180	RG	$_a33^g$	$_b28^g$	$_c12^g$	$_d0^a$
		IE%	63.33	38.88	86.6	100
	210	RG	$_a30^h$	$_b19^h$	$_c0^g$	$_d0^a$
		IE%	66.66	78.88	100	100

Different superscript and subscript letters are statistically different, according to the Duncan test at the level of significance of *P=0.05*

gave rise to a significant gradual repression in radial growth of *A. flavus* and accordingly the inhibition efficiency increased with the increase in ozone concentration. No mycelial growth was observed with 100% IE at 4 ppm at all exposure times.

Effect of ozone on lipid and tryptophan oxidation of A. flavus

There was a significant time dependent increase of malondialdehyde (MDA), a product of lipid oxidation, by *A. flavus* at 0-210 min, reaching maximum value (198 pmol mg-1) after 210 min exposure time (Fig. 4A). In this study, ozone caused oxidative degradation to proteins of *A. flavus*, as evaluated by the oxidation

Figure 2. **Viability of *C. albicans* spores exposed to different concentrations of ozone at different exposure times.**
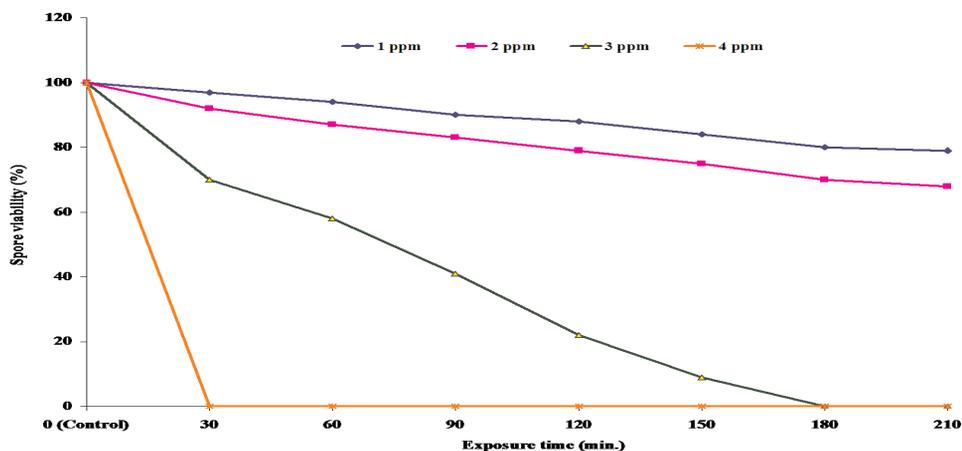
Figure 3. ***C. albicans* spores after exposure to optimum concentration of ozone (3 ppm) at different exposure times. Micrographs of *C. albicans* were taken using a light microscopy at 100× magnification.**
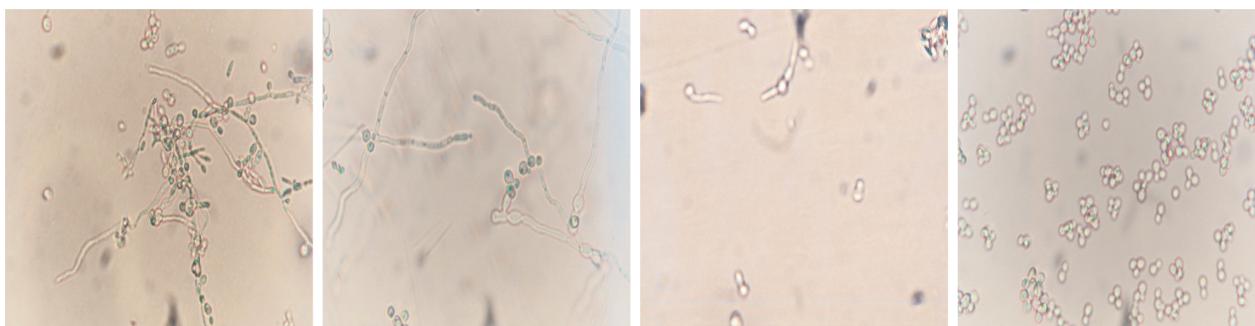
of tryptophan residues. This degradation was found to be a function of exposure time to ozone, as reflected by a decrease in fluorescence (Fig. 4B).

Effect of ozone on leakage of protein and nucleic acid in *A. flavus*

Figure 4C depicts that there was a statistically significant increase of protein content of *A. flavus* from 0-210 min. Furthermore, there was a time dependent increase in nucleic acid leakage reaching maximum values (0.49 and 0.56 mg/mL) of DNA and RNA after 210 min, respectively (Fig. 4D).

Efficacy of ozonation to degrade mycotoxins

In this experiment, two species selected from the isolated fungi were tested to determine the effect of their exposure to ozone on mycotoxin production. These species are well known as mycotoxin producers and include *Aspergillus flavus* and *Fusarium oxysporum*. Figure 5 shows the disappearance profile of trichothecene toxin at different ozone exposure times. The results proved that trichothecene was gradually degraded in a time dependent manner and disappeared at 180 min. Table 3 shows that *A. flavus* produced 3 aflatoxins (AFB1, AFB2, and AFG1). The production of aflatoxins by the test fungus gradually decreased with the extension of the exposure time to reach a minimum value for the mycelium originating from inoculum exposed for 180 minutes. In this

Figure 4. **Effect of optimum concentration of ozone (3 ppm) applied at different exposure times on lipid peroxidation (A), tryptophan oxidation (B), protein leakage (C), and nucleic acid leakage (D) of** *A. flavus*. **Bars are standard errors of triplicate determinations. Different letters indicate significance difference at *P*= 0.05.**
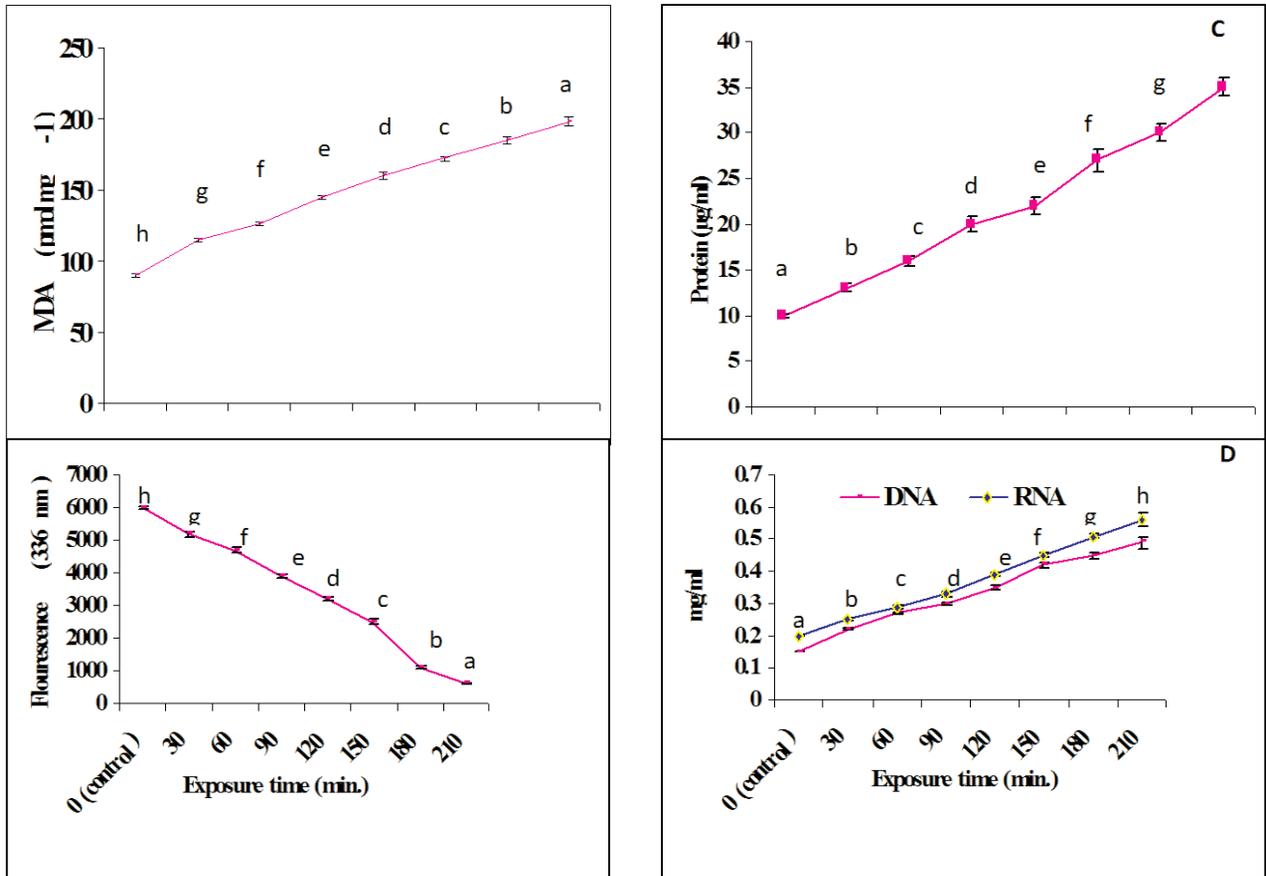

Figure 5. **Reductions, relative to starting material, in amount of trichothecene upon reaction with ozone (3 ppm) at different exposure times.**
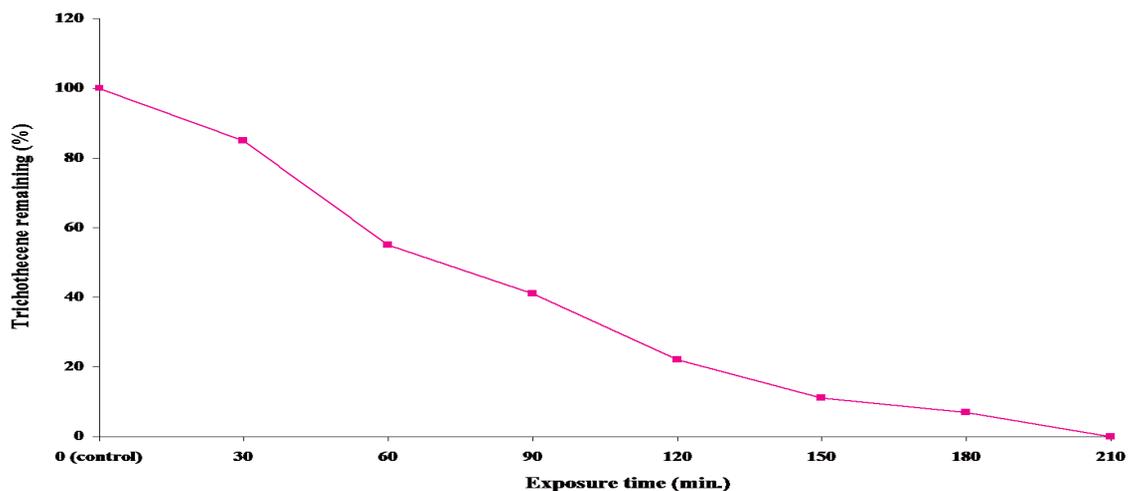
Table 3. **Effect of optimum concentration of ozone (3 ppm) applied at different exposure times on aflatoxin production (µg/g dry biomass) by *A. flavus***

Aflatoxin	Exposure time (min)								LSD at 0.05
	0	30	60	90	120	150	180	210	
AFB1	84.68	71.02	46.93	21.15	11.15	7.55	3.55	-*	10.75
AFB2	13.49	12.43	7.71	2.96	0.75	0.32	0.11	-	1.02
AFB1	1.82	1.73	1.12	0.02	0.02	0.0005	-	-	0.06
AFB2	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-
Total	99.90	85.18	55.76	24.13	11.92	7.87	3.66	-	11.82

LSD = least significant difference; *no mycelial growth

Table 4. **Wound reduction in control and treatment groups over 14 weeks**

Treatment period	Group					
	Control		Antifungal therapy		Ozone therapy	
	WSA	ARD%	WSA	ARD%	WSA	ARD%
Day 0	133.5±2.5	-	142±3	-	140±2.8	-
Week 2	125±2.0	6.3	121±2.8	14.78	111.5±2	20.35
Week 4	119.5±1.9	10.4	101±1.6	28.87	95.3±2.9	31.9
Week 6	113.2±2.3	15.2	97.5±1.4	31.33	79.5±1.2	43.21
Week 8	107.5±2.0	19.4	95.5±1.0	32.7	63.3±0.8	54.78
Week 10	101.3±2.7	24.11	91.3±1.5	35.7	33.5±0.8	76.07
Week 12	100±1.4	25.09	90.0±1.0	36.6	21.5±0.4	84.64
Week 14	98.7±1.0	26.06	88.5±1.5	37.6	9.5±0.1	93.27

WSA = wound surface area in cm^2; ARD% = area reduction percentage; WSA (day 0) = WSA (treatment)/WSA (day 0).

condition, the total aflatoxins accounted for 3.66 µg/g dry mass as compared to 99.90 µg/g dry mass under control conditions.

Therapeutic efficacy of ozone in patients with diabetic foot

As shown in Table 4, at the beginning of the study there were no significant differences among the three groups. At the end of treatments, a decrease in WSA was achieved in all groups. However, compared with the antifungal treated group, the ozone treated group reached a significant WSA decrease. By week 14, the WSA was reduced to 98.7, 88.5, and 9.5 cm^2 with the area reduction percentage (ARD %) of 26.06%, 37.6% and 93.27% in control group, antifungal group and ozone group, respectively. In 3 and 7 patients of control group and antifungal group, respectively, an increase in WSA was noticed, which did not occur in patients treated with ozone.

DISCUSSION

It has previously been suggested that fungal infections may be involved in the pathogenesis of DFU, but this has yet to be explored. Literature references on fungal infections of DFU are very scarce. Most reports describe a low incidence of fungal isolations or of ulcers probably infected by fungi or of ulceration improving upon systemic antifungal therapy (62-65).

Many studies have been performed on the incidence and continuum of bacterial infections and their effect on wound healing. However, the extent of fungal infections in diabetic foot wounds is an area, which has received insufficient interest. Our study showed a high prevalence and wide spectrum of fungi (10 different species) in DFU compared to previous studies. The isolates obtained from DFU in our study were similar to the spectrum of species isolated from blood stream samples by Gonzalez *et al*. (66). These results went in parallel with those obtained by

Mlinaric-Missoni (67), who report on the incidence of fungi in tissue biopsy specimens of 22 diabetic patients. The predominant isolates were *C. parapsilosis* (45.5%), *C. tropicalis* (22.7%), *C. albicans* (9.1%), and *C. glabrata* (9.1%). Bansal *et al*. (68) report isolation of fungi from swabs taken from 103 patients with diabetic foot wounds. The predominant species were *C. tropicalis* (29%), *C. albicans* (14%), *C. guilliemondii* (7%), followed by *A. flavus* (21%), *A. niger* (4%) and *Fusarium* species (14%). The study conducted by Chincholikar and Pal (65) showed the presence of various fungal pathogens in diabetic foot tissues, among which *Candida* species predominated. Heald *et al*. (62) have also reported the association of *Candida* species with ulceration in diabetic foot. The presence of *Trichosporon* species has been reported by Nair *et al*. (69). *A. flavus* and *F. solani* were observed in DFU by some authors (69-71). Özyrut *et al*. (72) report on a case of isolation of *F. sporotrichioides* from diabetic wound sample. The low incidence of pure *Candida* ulcer infections and the development of mold and yeast infections (mixed infections) in chronically infected ulcers indicate that *Candida* spp. play a secondary role in initiating DFU (67). A possible explanation of the direct relationship between the increased incidence of yeast infection of DFU and duration of foot ulcer infection is long wrapping of foot and application of antibiotics during treatment. Covering the skin with dressings that stimulate sweating and increase local temperature of the skin and the immunomodulating actions of antibiotics favor the growth and replication of yeasts (73-75).

Similarly, Mlinaric-Missoni *et al*. (67) report on yeast and fungal infections in diabetic foot ulcer biopsy when histopathologically examined, where numerous yeast conidia and hyphal fragments are seen. Nair *et al*. (69) report on the presence of mixed yeast- (66%) and mold-like (34%) culture in diabetic foot tissue. Warm, moist, and dark environment of foot in a shoe combined with improper immune system of diabetic patients in association with sweet foot may be the main reasons that make them more prone to fungal infection. The presence of sufficient moisture and nutrients in diabetic foot allow the fungi to reach macroscopic dimensions. Fungi are capable of extending fungal hyphae into the interior of diabetic foot, resulting in ulcer development and tissue necrosis.

The efficacy of ozone to suppress fungal sporulation is well documented in many reports (76-80). In a previous study carried out by us, we have reported that gaseous ozone at 3 ppm for 210 min completely inhibited sporulation of some fungi isolated from dried herbs and spices (50). Similarly, Sharpe *et al*. (51) showed that treatment with 450 ppb of ozone reduced *B. cinerea* spore viability by 99.5%. The results are consistent with those obtained by Margosan and Smilanick (81) who report that germination of *B. cinerea, Monilinia fruticola,* and *Penicillium digitatum* spores was inhibited by exposing them to high ozone concentration (1.30 µl/L) for 80 min. Heagle and Strickland (82) describe distortion and plasmolysis of conidia when exposed to 0.2 ppm ozone and suggest that ozone might enter directly the conidia or conidiophore. The spores most sensitive to ozone were, in general, relatively small and hyaline (50). Proteolysis of ozone to oxygen atoms could lead to the generation of hydroxyl radical (OH), a key reactive species during decomposition of spores (83).

The inhibition of mycelial growth of *Penicillium* on citrus fruit due to oxidizing action of ozone has been reported by Harding (79). Liew and Prange (84) found that ozone-enriched atmosphere delayed mycelial growth of *B. cinera* and *Sclerotinia sclerotiorum* on carrots. Similar effect on *Rhizopus stolonifer* was observed in grapes (85). It is believed that ozone, being a potent oxidant, may inactivate fungi by alteration of cell wall and/or protoplasmic components. Ozone may also inactivate microorganisms by causing damage to their genetic material. The antimicrobial activity of ozone has long been known. Less clear is its mode of action. Suggestions for primary targets include unsaturated lipids in the cell surface, enzyme sulfhydryl groups, nucleic acid, and others. Victorin (86) has affirmed that there are two mechanisms of ozone in the destruction of biomolecules. In the first mechanism, the ozone oxidizes sulfhydryl and amino acid groups of enzymes, proteins and peptides. In the second

mechanism, the gas acts as an oxidizing agent in the conversion of polyunsaturated fatty acids to peroxyacids. The differential activity of ozone against fungi might be due to the variation in their organic matter content, which may accelerate or reduce the toxicity of ozone (50). In this study, the tested fungi failed to grow at 4 ppm at all exposure times, consequently further experiments will be carried out at the significant optimum concentration (3 ppm).

Komanapalli and Lau (52) report that there is a significant leakage of MDA of *E. coli* exposed to ozone at 3-5 min. Ozone treatment resulted in the formation of oxidation products of lipids such as MDA (87). Ozone exposure results in the production of lipid peroxidation products (88). Ozone causes lipid peroxidation as a main cause of membrane deterioration (89). Ozone induced oxidation of endogenous lipids upon exposure to 0 and 100 ppb (90). Lipids are likely targets for attack by ozone (91). The position(s) of carbon-carbon double bonds in lipids can influence their structure and reactivity, so having a direct role in biological function using ozone-induced fragmentation (92).

Our results are consistent with those obtained by Kotiacho *et al*. (93) who state that methionine and tryptophan are the amino acids most easily oxidized by ozone. Results of experiments in which amino acids were exposed to ozone showed that the most susceptible amino acids were cysteine, tryptophan, methionine, and histidine (94-96). The most sensitive amino acid to ozone is tryptophan, which is degraded easily (30). Knight and Mudd (97) report that the aromatic amino acids such as tryptophan are oxidized by ozone causing degradation of the relative moiety. Ozone reacted with tryptophan and the reaction caused a decrease in the maximum fluorescence of the compound (98). The oxidation of tryptophans in proteins by ozone is markedly influenced by position in tertiary structure, position in membrane structure, and by chemical interactions within the protein (99). The oxidation of lipid and proteins has structural and functional roles in biomembranes, which could alter the ability to regulate permeability (52).

Our results are compatible with those of Gupta *et al*. (100) who state that there is a significant increase in the levels of protein RNA following exposure to ozone. Prolonged exposure to ozone (up to 30 min) leads to leakage of nucleic acid and lipid oxidation, which in turn affects membrane permeability (101). Ozone treatment influences the functional properties of protein as a result of structural changes by oxidation. However, the extent of change with ozone depends on the dose of ozone, treatment time, protein exposed to treatments, and environmental conditions (32). Feng *et al*. (102) found a significant induction of proteins after exposure to ozone for two days, where protein leakage gradually increased during the exposure period. Protein accumulation increased as the duration of exposure to ozone was prolonged (103). Ozone produces oxidative protein damage (104). FTIR spectroscopy is able to show some degree of oxidation of the protein only after prolonged exposure (105). Helices, sheets, coils and folded branches, which define the secondary and tertiary structures of protein, seem to be modified by the reaction of ozone with protein (32). The action of ozone converts the thiol group of cysteine into disulfides, which denature the protein and change its solubility (31).

It has been demonstrated that ozone reacts with unsaturated fatty acids in cell membranes to create secondary reactive species, such as aldehyde derivatives and lipid peroxides, which may reach the nuclei of intact cells and interact with DNA. However, the mechanism of DNA damage stimulated by ozone and/or its secondary reactive species remains mostly mysterious (106). Diaz-Liera *et al*. (107) suggest that DNA single-strand breaks induced by ozone may be mediated through H_2O_2 production. Ozone induced cleavages in deoxyribose phosphate backbone of double-stranded DNA, which proceeds through generation of hydroxyl radical (108). Our results are in harmony with those obtained by Cheng *et al*. (109), who found that ozone exposure at 80 ppb could induce increased oxidative DNA damage, including 8-oxoguanine and DNA single-strand breaks. The results are also in agreement with those reported by Cajigas *et al*. (110), who detected 5-deoxymethyluracil, thymine

glycol adduct generated by *in vitro* exposure to ozone. Relative reactivity of nucleobases of DNA with ozone was in the descending order GMP>UMP>CMP>AMP (111). Human bronchial cell lines had higher levels of DNA single-strand breaks after exposure to 100 and 200 ppb of ozone for 2 h (112). RNA of microorganisms is degraded into protein subunits by ozonation (113). DNA appears to be much more reactive toward ozone than RNA (30). Ozone reacts swiftly with RNA in solution, while in solid state, the reaction is slow but RNA is severely damaged (12). RNA has been found much less reactive toward ozone in the solid state than DNA (30). Chromosomal DNA may be one target of ozone degradation and its damage may be one of the factors responsible for cell damage.

Zorlugenc *et al*. (114) state that gaseous ozone treatment resulted in 48.77%, 72.39% and 95.21% aflatoxin reduction at 30, 60, and 180 min, respectively. The results of this study are also consonant with those of Young *et al*. (36), who report that aqueous ozone was shown to degrade a wide variety of trichothecenes to presumable simple products. Maeba *et al.* (115) confirmed destruction and detoxification of aflatoxins B_1 and G_1 with ozone. Aflatoxin B_1 and G_1 were sensitive to ozone and degraded with 1.1 mg/L of ozone in 5 min in model experiments. Inan *et al.* (33) state that the high oxidizing power of ozone achieved detoxification of aflatoxin. They subjected the contaminated samples to ozonation at various ozone concentrations (16, 33 and 66 mg/L) and exposure times (7.5, 15, 30 and 60 min) and recorded 80% and 93% reductions of the content of aflatoxin B_1 in flaked and chopped red peppers after exposures to 33 mg/L ozone and 66 mg/L ozone for 60 min, respectively. A reduction in the concentrations of total aflatoxins and aflatoxin B1 by approximately 30% and 25%, respectively, was observed for kernels exposed to ozone at 21 mg L^{-1} for 96 h (116). The olefinic position is one of the most reactive sites for reaction of ozone with organic compounds (117,118). Ozone is most likely to attack the double bond(s) in trichothecene (119). Young *et al*. (36) propose that the oxidation of some trichothecenes with ozone begins at the C-9,10 double bond with the addition of two atoms of oxygen, whereas the remainder of the molecule is not changed. Zotti *et al*. (120) observed discoloration of *A. flavus* colonies as a result of exposure to ozone. These authors attributed the discoloration to the destruction of pigments of the anthraquinone group, which are responsible for the yellow color of the species and are intermediaries needed for aflatoxin synthesis.

The results of this study are consistent with those of Martínez-Sánchez *et al*. (61), who report that patients treated with ozone achieved total recovery faster than patients treated with antimycotics. The superior efficacy of ozone in the treatment of DFU is due not only to its antimicrobial effect but also to its capacity to reduce hyperglycemia (20). The antidiabetic effect produced by ozone treatments seems to be associated with the antioxidant properties of ozone, increasing insulin sensitivity, and preventing oxidative stress associated with diabetes (61). Ozone treatment may promote oxidative preconditioning or adaptation to oxidative stress, preventing the damage induced by ROS (121,122). Ozone can be used with success in the treatment of chronic wounds such as trophic ulcers, ischemic ulcers and diabetic wounds (123,124). The application of ozonized oils (olive oil, sunflower oil) might be helpful in the treatment of foot ulcers in people with diabetes (125). The application of ozonized olive oil decreased the excretion of pus with no adverse side effects (21). The healing effect of ozone therapy likely results from biological, biorheological, and metabolic activities triggered by the exposure of blood to ozone.

CONCLUSION

Antifungal therapy is efficacious in infection, but not in colonization, and depends on the sensitivity of fungus species (problem of resistance). On the contrary, ozone therapy is better for colonization and in this case, ozone can prevent infection. Also, ozone can help support antifungal therapy during infection.

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