Original Contribution

Kurume Medical Journal, 64, 75-81, 2017

The Effect of Ozone on Colonic Epithelial Cells

HIDETOMO HIMURO

Department of Radiology and Department of Immunology, Kurume University of School of Medicine, Kurume 830-0011 Japan

Received 5 October 2017, accepted 14 November 2017
J-STAGE advance publication 21 May 2018

Edited by HIROHISA YANO

Summary: Due to its strong oxidation activity, ozone has been well known to kill bacteria and exert toxic effects on human tissues. At the same time, ozone is being used for the treatment of diseases such as inflammatory bowel disease in some European countries. However, the use of ozone for therapeutic purposes, despite its strong toxic effects, remains largely unexplored. Interestingly, we found that intrarectal administration of ozone gas induced transient colonic epithelial cell damage characterized by the impairment of cell survival pathways involved in DNA replication, cell cycle, and mismatch repair. However, the damaged cells were rapidly extruded from the epithelial layer, and appeared to immediately stimulate turnover of the epithelial layer in the colon. Therefore, it is possible that ozone gas is able to trigger damage-induced rapid regeneration of intestinal epithelial cells, and that this explains why ozone does not cause harmful or persistent damage in the colon.

Key words ozone, ozone therapy, colon epithelial cell, DNA repair, cell cycle

INTRODUCTION

Ozone is a pale blue unstable gas that is composed of three oxygen atoms [1-2]. Ozone has powerful oxidation activity that can damage cells and tissues [1-3]. Therefore, ozone, particularly at high concentrations, has toxic effects as well as strong bactericidal activity, making it useful for a broad range of sterilization purposes such as sanitization of water storage tanks [1-3]. Due to its oxidation activity, the harmful effects of ozone on the human body are well known [4-5]. However, ozone, when used at appropriate concentrations, has been applied in the field of alternative medicine, particularly in some European countries, for the treatment of inflammatory bowel disease, atherosclerosis, ischemic heart disease, diabetic nephropathy, skin ulcer, dermatosis, infection, viral disease, and cancer [1-3, 6-16]. In addition, various approaches have been used to administer ozone, including intrarectal or subcutaneous administration, autohemotherapy, in which the patient's blood is ozonated outside the body, *in vitro*, and then intravenously or intramuscularly readministered to the patient (MAH; Major Autohemotherapy), and ointments of ozoneated water or cream [17-20]. However, it remains obscure why ozone, despite its strong toxic effects, should be useful in the treatment of some diseases.

Since ozone has generally been believed to possess a strong toxic effect, we initiated this study to dissect out how ozone provides the toxic effect on the epithelial cells. However, we unexpectedly found that the toxic effect of ozone is transient and ozone rather induces prompt regeneration. This finding provides the possibility of leading to the elucidation of the mechanism of the therapeutic effect of ozone therapy.

METHOD

Mice

C57BL/6 mice were maintained in a semi-SPF facility in which *Pasteurella pneumotropica* and *Trichomonas* spp. were present at Kurume University

Corresponding Author: Hidetomo Himuro, MD, Department of Radiology and Department of Immunology, Kurume University School of Medicine, 67 Asahi-machi, Kurume-city, Fukuoka, 830-0011. TEL: +81-942-31-7576, FAX: +81-942-32-9405, E-mail: hidetomo_himuro@med.kurume-u.ac.jp

Abbreviations: MAH; Major Autohemotherapy

School of Medicine. All animal experiments were performed in accordance with guidelines approved by the Ethics Committee of Kurume University.

Intrarectal administration of ozone:

Enema using 50% w/v polyethylene glycol 3,350 was performed to remove intestinal contents, after which 5 ml of ozone gas (20 μg/ml) generated by an ozone purifier (Hensler company) was intrarectally administrated in 10 seconds.

Isolation of epithelial cells:

Epithelial cells were isolated as a crypt unit as previously described [21-23]. Briefly, mice were anesthetized by inhalation of isoflurane and an incision was made in the abdomen. A 21-gauge needle was inserted in the proximal part of the colon and the intestinal content was flushed out by phosphate buffer solution (PBS). After opening the thoracic cavity, a catheter was inserted into the left ventricle and perfusion was performed using 10 mL of 37°C 30 mmol/L EDTA in Hank's balanced salt solution (HBSS). At the end of the perfusion, the entire colon except for the cecum was removed, inverted, and shaken in 2mM EDTA at 2500 rpm for 30 seconds by mini beater (Biospec-Products, Bartlesville, OK). The tissue remnants were discarded and the epithelial crypts in the supernatant were allowed to settle to the bottom of the tube and then washed with cold PBS.

Gene expression analyses

Total RNA was isolated from purified colonic epithelial cells using TRIzol Reagent (Ambion by Life Technology). RNA was subjected to DNA microarray analysis (SurePrint G3 Mouse Gene Expression Microarray 8 × 60K v2) using a Cell Innovator (http://www.cell-innovator.com/). Briefly, cRNA was amplified, labeled, and hybridized to a 60K Agilent 60-mer oligo microarray. All hybridized microarray slides were scanned by an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1). Raw signal intensities and flags for each probe were calculated from hybridization intensities (gProcessedSignal), and spot information (gIsSaturated, etc.), according to the procedures recommended by Agilent [24, 25]. We selected probes that assigned a present 'P' flag to at least one sample, excluding lincRNA probes. To identify up or down-regulated genes, we calculated Z-scores and ratios (non-log scaled fold-change) from the normalized signal intensities of each probe for comparison between control and experiment samples [26].

Histological Assessment

Colon samples were fixed in 10% formalin solution. Paraffin-embedded tissue sections were stained with H&E, using standard techniques.

5-Bromo-2=-Deoxyuridine Incorporation

5-bromo-2=-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO), at a concentration of 5 mg/200 L PBS, was injected intraperitoneally 1 hour before sacrifice. Paraffin-embedded sections were stained with anti-BrdU antibody (SeroTec, Oxford, England) and BrdU-incorporated CECs per crypt units were counted using 40 different high power fields.

RESULT

Inhibition of DNA replication and proliferation pathways in the intestinal epithelial cells by ozone; Intrarectal administration of ozone gas has been applied for treatment of several diseases such as infectious enteritis, ulcerative colitis, Crohn's disease and fistulas, ischemic enterocolitis, allergic diseases, autoimmune diseases, and cancer. It is widely used mainly as an alternative method of MAH. [1-3]. Concerns have been raised that this therapeutic regimen using ozone, which certainly possesses oxidation-dependent toxicity, may have a harmful effect on intestinal tissues. To address this issue, DNA microarray analysis was initially employed to comprehensively evaluate the molecular pathways induced by ozone exposure. To do so, we intrarectally administrated a relatively high dose of ozone gas (20 μg/ml, 5 ml) and 5 ml of air as control into C57BL/6 mice in 10 seconds. To determine the toxicity of ozone, a lethal dose of ozone was used. Since epithelial cells, which cover the colonic tissue, are first and directly exposed to the intrarectally administrated ozone gas, we isolated epithelial cells using the 30 mM perfusion method at 6 hours after the administration. The purified epithelial cells isolated as a crypt unit were then subjected to RNA isolation and DNA microarray analysis.

DNA microarray analysis showed that some restricted pathways are selectively inhibited by ozone exposure, including DNA replication, cell cycle, mismatch repair, P53 signaling, nucleotide excision repair, and Foxo signaling pathways (Table 1). Among these pathways, the DNA replication pathway was most significantly impaired by ozone exposure as indicated by p value 1.8×10^{-19}. The pathway was composed of licensing factors, such as Mini-chromosome mainte-

TABLE 1.
Major molecular pathways impaired by ozone gas exposure: The impaired molecular pathways in the purified colonic epithelial cells after ozone gas exposure as compared to air exposure was analyzed using a Pathway analysis software (kegg). List of pathways that are dominantly affected at 6 hours after ozone exposure are shown. "Count" represents the number of differentially expressed genes.

Pathway	count	P-value
DNA replication	23	1.8×10^{-19}
Cell cycle	35	4.3×10^{-15}
Mismatch repair	10	9.5×10^{-5}
P53 signaling pathway	14	2.7×10^{-3}
Nucleotic excision repair	11	3.1×10^{-3}
FoxO signaling pathway	15	2.0×10^{-2}

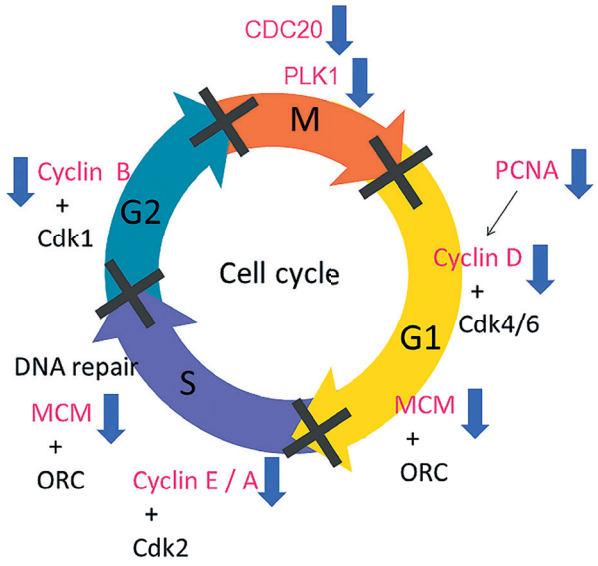


Fig. 1. Schema of functions mediated by the genes affected by ozone exposure. Molecules (cyclin genes and MCM), which are indispensable for the progression of the cell cycle, are downregulated. In addition, molecules (SFN, GAGDD45, PCNA), which serve as the negative regulators of above molecules, were upregulated. Red arrows indicate the upregulated molecules and blue arrows indicate downregulated molecules.

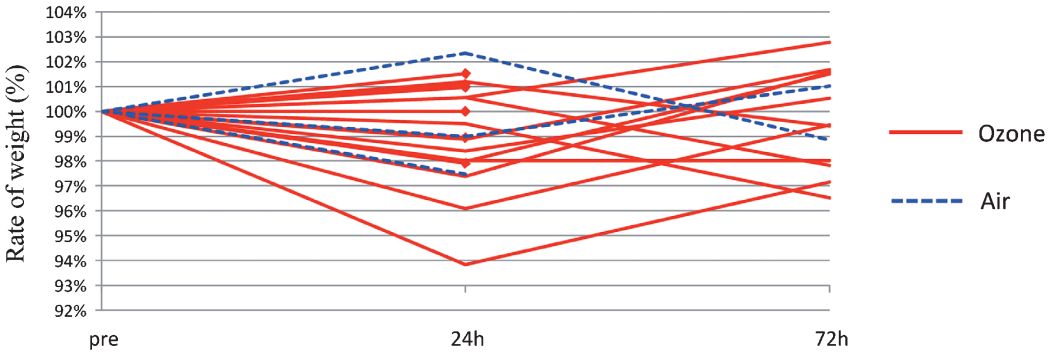
nance complex and cyclin gene, and is indispensable for DNA replication. [24, 25]. A second pathway that was strongly affected by ozone exposure was the cell cycle cascade ($P=4.3\times10^{-15}$) composed of DNA polymerase α-primase complex and MCM complex (helicase). Interestingly, molecules involved in the entire (M, G1, S and G2 phases), but not a selected, phase of cell cycle were simultaneously down regulated in colonic epithelial cells by ozone exposure. [Figure 1] These findings suggest that intrarectal administration of ozone gas impairs the fundamental signaling cascades necessary for cell survival of the colonic epithelial cells.

No lethal damage by intrarectal exposure of ozone gas: Since intrarectal administration of ozone gas induced significant alteration of major signaling pathways associated with lethal damage to colonic epithelial cells, we initially hypothesized that a majority of mice would be unable to survive after intrarectal ozone exposure. Unexpectedly, there was, however, no significant difference in the fatality rate in mice exposed to ozone versus air (Table 2). In addition, no obvious clinical symptoms such as diarrhea or body weight loss were observed in the mice exposed to ozone as compared to control mice exposed to air (Table 2). These clinical findings were also supported by histological findings that showed no obvious difference in inflammation of the colon between ozone- and air-treated mouse groups at 6 hours, 24 hours and 72 hours after administration. These unexpected findings suggest that intrarectal administration of ozone gas, although it induces cell damage in colonic epithelial cells, is insufficient to induce the lethal tissue damage in the colon.

Rapid extrusion of damaged epithelial cells by exposure of ozone gas: Since the absence of lethal tissue damage by ozone exposure was unexpected, we next examined the homeostatic change of epithelial cells that were directly exposed to ozone gas. Intact epithelial barrier integrity is maintained by a mechanism of cell extrusion that sheds (removes) cells from the epithelial layer [26-27]. Interestingly, enhanced cell extrusion from the epithelial layer was seen in the colon around 6 hours after exposure to ozone gas

TABLE 2.
No symptom after ozone gas exposure: The Table shows the percentage of mice that died after exposure of ozone versus air, and he percentage of mice that exhibited clinical symptom as indicated by diarrhea. The Fig shows the body weight change. ◆:used for experiments.

Sympton	Air(control) : 10	Ozone : 23
diarrhea	0 (0/10)	0 (0/10)
decrease activity	0 (0/10)	0 (0/10)
body weight loss	0 (0/10)	0 (0/10)
death	10 (1/10)	4.3 (1/23)



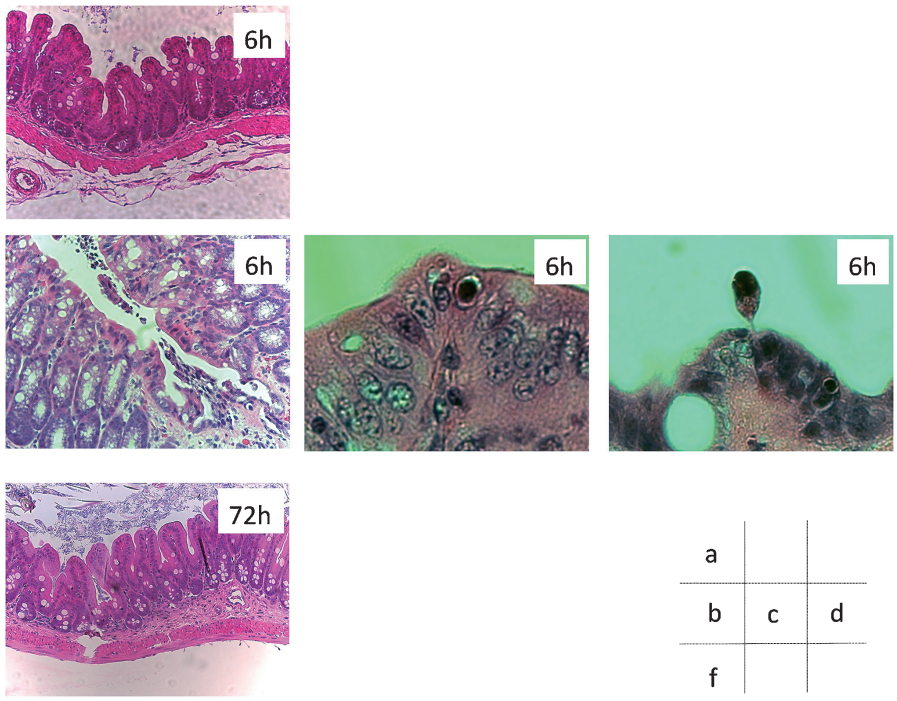


Fig. 2. Histological change of colonic epithelial cell by ozone gas exposure.
The colon was evaluated by H&E staining at 6hr after air administration (original magnification 20×) (a), at 6hr after ozone gas administration (b x20, c x40, d x40) and at 72hr after ozone administration (x 20) (e) Enhanced extrusion of apoptotic or necroptotic cells from the colonic epithelial layer is seen in b, c, d. No obvious alteration is seen in the colonic epithelial layer (e) at 72 hr after ozone gas exposure.

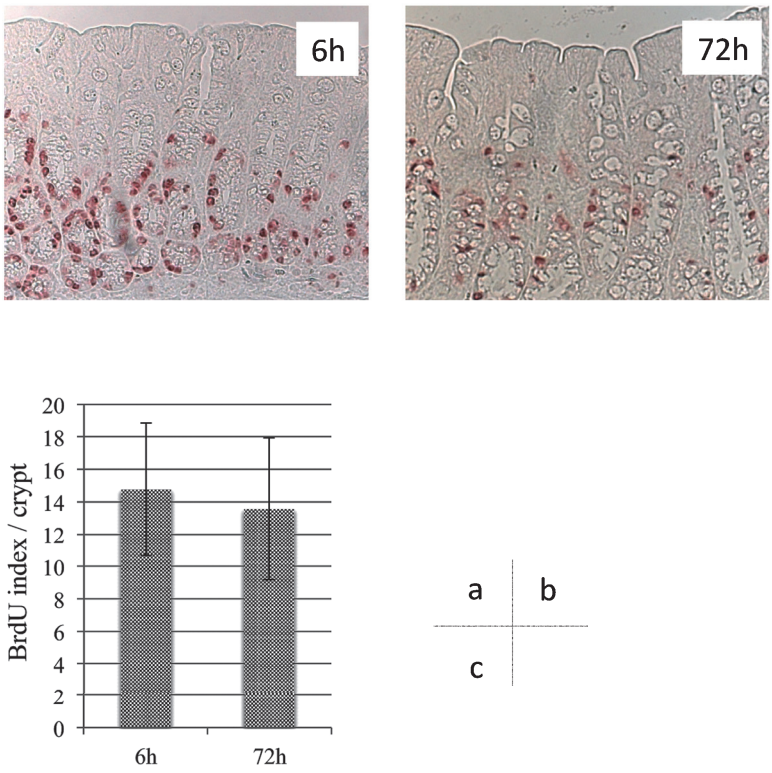


Fig. 3. Proliferative response of colonic epithelial cells at 6,24hr after ozone gas exposure: a-b : Immunohistochemical analysis shows the Incorporation of Brdu (original magnification 40×) in the colon. c : The BrdU –incorporated cells per a crypt was counted, and the average of BrdU-incorporated cells per a crypt is shown in .

(about Fig. 2 d). The shed epithelial cells were characterized by apoptotic or necroptotic morphology as indicated by condensed and fragmented nuclei (Fig. 2 b-d). Within 72 hours after ozone exposure, however, the colonic epithelial layer exhibited normal morphology, suggesting a rapid recovery of epithelial layer from ozone-induced damage (Fig. 2 e). Since epithelial regeneration is closely associated with the proliferative responses, we next examined epithelial cell proliferation by evaluating BrdU-incorporation [31]. Unexpectedly, there was no significant difference in the proliferative response of colonic epithelial cells in mice after intrarectal administration of ozone gas versus air (Fig. 3). Therefore, this finding raises the possibility that epithelial cells damaged by ozone are rapidly removed by a cell extrusion mechanism but epithelial cell regeneration characterized by proliferation is not induced.

DISCUSSION

Ozone, although it possesses toxic effects through oxidization of cells, has been applied for therapeutic purposes in a number of disease conditions such as for promoting skin wound healing. However, the mechanism by which ozone stimulates the wound healing process has been largely unexplored. [28, 29] In this study we found that intrarectal administration of ozone gas induces transient epithelial cell damage caused particularly by the impairment of DNA replication and cell cycle pathways. However, an extrusion of damaged cells from epithelial layer was rapidly induced within 6 hours after ozone exposure, which may immediately stimulate turnover of the epithelial layer in the colon. The fact that ozone's effect is transient due to its instability, and that ozone gas is not retained within the administered area, may allow ozone gas to initiate damage-induced rapid regeneration of intestinal epithelial cells.

Although ozone induced major signaling pathways involved in cell damage in the colonic epithelial cells that provide the first line of defense against enteric bacteria, no lethal inflammation was elicited by intrarectal administration of a relatively high dose of

ozone gas. Ozone has a strong bactericidal effect and has been widely used for sterilization in various fields such as sanitization of drinking water [1-3]. Therefore, it is possible that decontamination of enteric bacteria by ozone may contribute to the inhibition of lethal inflammation, indicating a second potential beneficial property of ozone, along with induced regeneration of the epithelial layer.

Infectious colitis is a common disease that can be treated by antibiotics. However, there are still some pathogens such as Carbapenem-resistant *enterobacteriaceae, Clostridium difficile* that are difficult to treat. [31, 32] Ozone has not only bactericidal capacity but also an ability to induce a rapid turnover of epithelial cells, which may facilitate removal of infected epithelial cells. Therefore, it is possible that intrarectal administration of ozone gas may be applicable for patients with infectious colitis resistant to conventional therapies. In addition, as indicated, some reports have suggested the beneficial effect of ozone on inflammatory bowel disease. Since reinforcement of epithelial barrier function contributes for the improvement of intestinal inflammation, it is possible that the rapid turnover of epithelial cells by the administration of ozone gas may reinforce epithelial barrier function, leading to improvement of intestinal inflammation.

ACKNOWLEDGMENTS: This work was supported by Ozone Therapy Research Institute, Japan / Ozonosan Japan Co., Ltd. This work was supported by JSPS KAKENHI Grant Number JP90772567. I very much thank Professor Toshi Abe and Professor Etsuyo Ogou for their support.

REFERENCES

1. Bocci V. Biological and clinical effects of ozone: Has ozone therapy a future in medicine? Br J Biomed Sci. 1999; 56:270-279.
2. A. M. Elvis and J. S. Ekta. Ozone therapy: A clinical review J Nat Sci Biol Med. 2011 Jan-Jun; 2(1): 66-70.
3. Bocci V, Borrelli E, Travagli V and Zanardi I. The ozone paradox: ozone is a strong oxidant as well as a medical drug. Med Res Rev. 2009 Jul; 29(4):646-682.
4. Rediguieri CF, De Bank PA, Zanin MHA, Pinto TJA. et al : The effect of ozone gas sterilization on the properties and cell compatibility of electrospun polycaprolactone scaffolds. J Biomater Sci Polym Ed. 2017 Nov; 28(16):1918-1934.
5. Bocci V. Is it true that ozone is always toxic? The end of a dogma. Toxicol Appl Pharmacol. 2006; 16:493-504.
6. Mustafa MG. Biochemical basis of ozone toxicity. Free Radical Biol Med. 1990; 9:245-265.
7. Hernández F, Menéndez S, and Wong R. Decrease of blood cholesterol and stimulation of antioxidative response in cardiopathy patients treated with endovenous ozone therapy. Free Radical Biol Med. 1995; 19:115-119.
8. Bocci V, Zanardi I, Huijberts MS, and Travagli V. Diabetes and chronic oxidative stress. A perspective based on the possible usefulness of ozone therapy. Diabetes and Metabolic Syndrom. 2011; 5:45-49.
9. Bocci V. Ozonization of blood for the therapy of viral diseases and immunodeficiencies: A hypothesis. Med Hypothesis. 1992; 39:30-34.
10. Wells KH, Latino J, Gavalchin J, and Poiesz BJ. Inactivation of human immunodeficiency virus type 1 by ozone in vitro. Blood. 1991; 78:1882-1890.
11. Carpendale MT, and Freeberg JK. Ozone inactivates HIV at noncytotoxic concentrations. Antiviral Res. 1991; 16:281-292.
12. Washutti J, Viebahn R, and Steiner I. The influence of ozone on tumor tissue in comparison with healthy tissue. Ozone Sci Engg. 1990; 12:65-72.
13. Bocci V, and Di Paolo N. Oxygen-ozone therapy in medicine: an update. Blood Purif. 2009; 28(4):373-376.
14. Demirbag S, Uysal B, Guven A, Cayci T, Ozler M et al. Effects of medical ozone therapy on acetaminophen-induced nephrotoxicity in rats. Ren Fail. 2010; 32(4):493-497.
15. Morsy MD, Hassan WN, and Zalat SI. Improvement of renal oxidative stress markers after ozone administration in diabetic nephropathy in rats. Diabetol Metab Syndr. 2010; 13(2(1)):29.
16. Luongo M, Brigida AL, Mascolo L, and Gaudino G. Possible Therapeutic Effects of Ozone Mixture on Hypoxia in Tumor Development. Anticancer Res. 2017 Feb; 37(2):425-435.
17. Sunnen GV. Ozone in Medicine: Overview and Future Directions http://www.triroc.com/sunnen/topics/ozonemed.htm 2017.9.1
18. Rilling S: The basic clinical applications of ozone therapy. Ozonachrichten 1985; 4:7-17.
19. Wolff H. Aktuelles in der ozontherapy. Erfarhr hk 1977; 26:193-196.
20. Turk R. Ozone in dental medicine. Ozonachrichten 1985; 4:61-65.
21. Bjerknes A, and Cheng H. Methods for the isolation of intact epithelium from the mouse intestine. Anat Res. 1989; 199:565-574.
22. Mizoguchi E, Mizoguchi A, Takedatsu H, Cario E, de Jong YP et al. Role of tumor necrosis factor receptor 2 (TNFR2) in colonic epithelial hyperplasia and chronic intestinal inflammation. Gastroenterology. 2002; 122:134-144.
23. Mizoguchi E, Xavier RJ, Reinecker HC, Uchino H, Bhan AK et al. Colonic epithelial functional phenotype varies with type and phase of experimental colitis. Gastroenterology. 2003; 125:148-161.
24. Sclafani RA, and Holzen TM. Cell cycle regulation of DNA replication. Annu Rev Genet. 2007; 41:237-280.
25. Bell SP and Dutta A. DNA Replication in Eukaryotic Cells. Ann Rev Biochemistry 2002; 71:333-374.
26. Günther C, Neumann H, Neurath MF, and Becker C. Apoptosis, necrosis and necroptosis: cell death regulation in the intestinal epithelium. Gut. 2013; 62(7):1062-1071.
27. Eisenhoffer GT, Loftus PD, Yoshigi M, Otsuna H, Chien CB et al. Crowding induces live cell extrusion to maintain homeostatic cell numbers in epithelia. Nature. 2012;

484(7395):546-549.
28. Valacchi G, Sticozzi C, Zanardi I, Belmonte G, Cervellati F et al. Ozone mediators effect on “in vitro” scratch wound closure. Free Radic Res. 2016 Sep; 50(9):1022-1031.
29. Xiao W, Tang H, Wu M, Xu X et al. Ozone oil promotes wound healing by increasing the migration of fibroblasts via PI3K/Akt/mTOR signaling pathway. Biosci Rep. 2017 Sep 1.
30. Neil Gupta, Brandi M. Limbago, Jean B. Patel, and Alexander J. Kallen. Carbapenem-Resistant Enterobacteriaceae: Epidemiology and Prevention, Clinical Infectious Diseases, 2011; 53(1):60-67.
31. Lessa FC, Winston LG, and McDonald LC. Emerging Infections Program C. difficile Surveillance Team. Burden of Clostridium difficile infection in the United States. N Engl J Med. 2015; 372:2369-2370.
32. Loo VG, Poirier L, Miller MA, Oughton M, Libman MD et al. A predominantly clonal multi-institutional outbreak of Clostridium difficile-associated diarrhea with high morbidity and mortality. N Engl J Med. 2005; 353:2442-2449.