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Summary

Although ozone therapy is extensively applied when wound repair and antimicrobial effect are necessary, little is known about cellular mechanisms regarding this process. Thus, this study aimed to evaluate ozone cytotoxicity in fibroblasts (L929) and keratinocytes (HaCat) cell lines, its effects on cell migration and its antimicrobial activity. Cells were treated with ozonated phosphate-buffered saline (8, 4, 2, 1, 0.5 and 0.25 μg/mL ozone), chlorhexidine 0.2% or buffered-solution, and cell viability was determined through MTT assay. The effect of ozone on cell migration was evaluated through scratch wound healing and transwell migration assays. The minimum inhibitory concentrations for *Candida albicans* and *Staphylococcus aureus* were determined. Ozone showed no cytotoxicity for the cell lines, while chlorhexidine markedly reduced cell viability. Although no significant difference between control and ozone-treated cells was observed in the scratch assay, a considerable increase in fibroblasts migration was noticed on cells treated with 8 μg/mL ozonated solution. Ozone alone did not inhibit growth of microorganisms; however, its association with chlorhexidine resulted in antimicrobial activity. This study confirms the wound healing and antimicrobial potential of ozone therapy and presents the need for studies to elucidate the molecular mechanisms through which it exerts such biological effects.

Keywords: Ozone therapy; cytotoxicity; wound healing; cell migration; antimicrobial effect.

INTRODUCTION

Ozone has been medically used for over 150 years, being applied throughout these years as either a disinfection method or a treatment option for several diseases (*Elvis and Ekta*, 2011), including chronic inflammatory conditions (*Bocci et al.*, 2015), diabetic foot ulcers (*Liu et al.*, 2015), osteonecrosis (*Fliefel et al.*, 2015), periodontal disease (*Gupta and Mansi*, 2012) and dental caries (*Samuel et al.*, 2016), among others. Such a wide use is justified by its broad biological applications, which cover a potential antimicrobial effect, as well as the activation of the immune system and the induction of wound healing (*Elvis and Ekta*, 2011, *Zanardi et al.*, 2016).

Ozone is a triatomic molecule consisting of three oxygen atoms (O_3). It is an unstable structure, decomposing to molecular oxygen (O_2) and an atomic oxygen, which is highly reactive (*Stockburger*, 2002). Three fundamental forms of topical ozone application are described: ozonated water, ozonated oil and oxygen/ozone gas (*Saini*, 2011). For treatment of systemic conditions, the ozonated autohemotherapy (O_3-AHT) is the form of choice (*Sagai and Bocci*, 2011). In contrast with conventional medical therapeutic modalities, ozone therapy is quite economical and may lead to a marked reduction of both medical costs and adversities.

Wound healing is a multiphase process that consists of three overlapping but distinct stages: inflammation, tissue proliferation and remodeling. It begins at the moment of injury, with the formation of the blood clot responsible for the hemostasis and for cell recruitment. Neutrophils are the first cells to arrive, preventing bacterial infection and activating keratinocytes, fibroblasts and immune cells. After 2-3 days, monocytes arrive and differentiate into macrophages, debriding necrotic tissue and controlling bacteria alongside with neutrophils. The proliferation stage begins 2-10 days after injury, when macrophages adopt an anti-inflammatory, profibrotic phenotype,

producing growth factors such as transforming growth factor-β (TGF-β) and recruiting fibroblasts from the wound borders. These cells form the granulation tissue, which functions as a substrate for keratinocyte migration, proliferation and differentiation during the re-epithelialization phase. Remodeling of the extracellular matrix (ECM) begins after 2-3 weeks after injury, and lasts for a year or more. In this stage, the cellular content of the wound decreases, and collagen fibers of the ECM are substituted and reorganized into a stronger network, even though the tissue never regains the properties of its uninjured counterpart (*Gurtner et al.*, 2008, *Zielins et al.*, 2014).

Ozone therapy, mostly in one of its topical forms, has been clinically used as an aid to wound healing. This practice is justified by the oxidative killing effect that ozone has on microorganisms such as *Candida albicans* (*Khatri et al.*, 2015), a fungus present mostly in oral and vaginal cavities, and *Staphylococcus aureus* (*Al-Saadi et al.*, 2015), bacteria commonly implicated in wound infections, controlling wound contamination and reducing the time required for proper healing (*Travagli et al.*, 2010). A complete eradication of *S. aureus* was observed on planktonic cells and biofilm after a respective 60- and 360-minute continuous exposure to ozonated saline solution (*Al-Saadi et al.*, 2015).

The increase in O_2 tension within the wound site also justifies the use of ozone, considering that it enhances the formation of granulation tissue, accelerating the wound closure (*Travagli et al.*, 2010). Additionally, a higher expression of growth factors TGF-β and vascular endothelial growth factor (VEGF), which play important roles in the wound repair process, is described in both clinical and experimental wound healing studies with ozone therapy (*Valacchi et al.*, 2011, *Zhang et al.*, 2014), and endorses its use in such conditions.

Even though ozone therapy is extensively applied in situations in which a wound

repair and antimicrobial effect are necessary, not much is known about the cellular mechanisms regarding this process. In an effort to understand how cells react to this therapeutic option, this study aimed to evaluate the cytotoxicity of ozone in fibroblast and keratinocyte cell lines, its effects on cell migration and wound healing and its antimicrobial activity.

MATERIALS AND METHODS

Cell lines, strains and culture conditions

A human keratinocyte cell line (HaCaT) and a mouse fibroblast cell line (L-929), described in ATCC (American Type Culture Collection), were used in the experiments. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin-streptomycin) and maintained at 37°C and 5% CO_2. For all experiments, cells were detached with trypsin (0.25%)/ethylenediaminetetraacetic acid (EDTA; 1 mM) solution. Cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antimicrobial assays were performed with *Candida albicans* (ATCC 10231) and *Staphylococcus aureus* (ATCC 29213) strains. All microorganisms were obtained from ATCC and provided by Oswaldo Cruz Foundation (FIOCRUZ, Brazil). Cultures were stored at −80ºC in Mueller Hinton medium (Himedia, Mumbai, India) containing glycerol, and they were properly regenerated before use.

Cell viability assay—MTT

HaCaT and L-929 cells were seeded at the density of $5x10^3$ cells/well into 96-well plates and incubated overnight in conditions previously described. Cells were treated with serial dilutions of ozonated phosphate-buffered saline (PBS) solution (8, 4,

2, 1, 0.5 and 0.25 µg/mL), chlorhexidine 0.2% (positive control) or PBS alone (negative control). For PBS ozonation at 8 µg/mL, 1 L PBS was applied to the O&L1.5 RM ozone generator (Ozone & Life, São José dos Campos, Brazil) for 10 minutes, with an oxygen flux of ¼ L/min. Contact time was 5 minutes, after which the solution was replaced with culture medium. Chlorhexidine was diluted directly in culture medium. For cells undergoing a 48-h experiment, treatment was repeated after 24 h. After 24 or 48 h, 10 µL of MTT solution (3,[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA) at 5 mg/mL was added to each well, followed by incubation for 4 h at 37°C. After incubation, medium was discarded and 100 µL of acidified isopropanol was added to dissolve the formazan crystals. Absorbance was measured at 570 nm in a Beckman Coulter reader.

Scratch wound healing assay

Cells were plated at $1x10^6$ cells/well into fibronectin-coated 6-well plates. The cells monolayer was scratched manually with a yellow plastic pipette tip, washed with PBS and treated with 8 µg/mL ozone solution or control (PBS) for 5 minutes, then replaced with culture medium. An inverted microscope (Zeiss Primo Vert, Göttingen, Germany) equipped with a digital camera (Zeiss ERc 5s, Göttingen, Germany) was used to obtain images of the wound in different times of treatment at 10x magnification. Considering the specific growth pattern of each cell line, the wound closure for L929 was monitored during 24 h counting from hour zero, until the borders of the wound could no longer be identified. For HaCaT, the measurements were made during 96 h, regardless of a complete closure at this time point. Wound closure was measured by the wound area in each period and expressed as percentage of the initial wound area at hour zero, with ozone compared to control.

Transwell migration assay

The 6.5-mm transwell chambers (Corning Costar, Cambridge, MA, USA), with polycarbonate membrane inserts (8-µm pore size), were placed in 24-well plates containing 600 µL of plain DMEM per well. Cells ($2x10^4$) were seeded into the upper compartment and incubated at 37ºC for 24 h. After this period, cells were treated with ozone at 8 µg/mL or PBS at the upper compartment for 5 min, after which the treatment was replaced with culture medium. At 72 h after treatment, the cells that had migrated through the membrane to the lower compartment were fixed in methanol for 20 minutes, incubated with 0.2% Crystal Violet dye for 5 min, and washed with PBS 10 times. After the last wash, the stained cells were photographed at 4x magnification. Migrated cells were counted with ZEN image analysis software (Carl Zeiss Microscopy GmbH, Göttingen, Germany).

Antimicrobial susceptibility assay with microdilution method

Antimicrobial testing to determine minimum inhibitory concentrations (MIC) was performed using broth microdilution protocols defined by the CLSI (Clinical Laboratory Standards Institute): M-27A2 for yeast and M7-A6 for bacteria. The spectrophotometric method for inoculum preparation was used to obtain appropriate densities of *C. albicans* in the Sabouraud Dextrose medium (Himedia, Mumbai, India) and of *S. aureus* in the Mueller Hinton medium. *C. albicans* yeast suspensions at 10^6 CFU/mL were obtained using turbidity standard 0.5 McFarland, by adjusting the optical density of the samples with saline solution 0.85% and measuring absorbance at 530 nm. *S. aureus* suspensions at 10^8 CFU/mL were obtained using saline solution 0.9% and measuring absorbance at 625 nm. The assays were performed in 96-well plates containing 100 µL of inoculum to which was added 100 µL of 8-0.0039 µg/mL

ozonated PBS, 2-0.0009% chlorhexidine or a combination of 2-0.0009% chlorhexidine and 8 µg/mL ozone. After 24 h of treatment, 30 µL of 0.01% resazurin (Steinheim, Germany) was added to each well, followed by a 4-h incubation at 37ºC. Growth was indicated by a reddish-pink color. MIC was determined as the lowest concentration of treatments able to visually inhibit microorganism growth. Wells free of microorganisms were considered as negative control, and wells with microorganisms alone were used as growth control.

Statistical analysis

Statistical analysis was performed on the means or medians of triplicates that resulted from three independent replications of each experiment. For MTT assay, the Kruskal-Wallis test with the Dunn's post-test was used. For the scratch wound healing assay, one-way analysis of variance with the Tukey post-test was used. For transwell migration assay, the Student t-test was used. Statistical analyses were performed with GraphPad Prism software, version 5.0. A threshold of $p<0.05$ was defined as statistically significant.

RESULTS

The results for cell viability after treatment with ozone or chlorhexidine are showed in Figure 1. Chlorhexidine at a 0.2% concentration, used as positive control, resulted in marked cell viability reduction in both cell lines. For keratinocytes, the HaCaT cell line, the median viability rates were 38% and 30% for 24 h and 48 h, respectively. For fibroblasts, the L929 cell line, these rates were of 15% and 28%. Such

reductions were considered statistically significant, and all rates were less than 50%, indicating a potential cytotoxic effect of chlorhexidine on HaCaT and L929 cells.

For the 24-h treatment, HaCaT cells treated with ozonated saline showed at all concentrations a slight increase in cell viability when compared to control. The 8 µg/mL concentration resulted in the highest increase for this cell line, with a cell median viability rate of 134%. Interestingly, after 48 hours of treatment, a decrease in cell viability was observed for ozone concentrations of 0.5 µg/mL or higher, with viability rates varying from 69% (2 and 4 µg/mL) to 85% (1 µg/mL). Nevertheless, none of these results was considered statistically significant.

In a different manner, the treatment with ozonated saline resulted in reduced L929 cell viability with both 24-h and 48-h treatments, whenever it was compared to negative control. The only exception was observed with the 8-µg/mL concentration after 24 h of treatment, which resulted in a median viability rate of 106%, comparable to negative control. For the 48-h treatment with ozonated saline, cell viability rates varied from 43% (4 µg/mL) to 97% (0.5 µg/mL). Despite the aforementioned variations on cell viability rates, no results were considered significant.

Considering the cell viability rates resulting from treatment with each specific ozone concentration on HaCaT or L929 cell lines, no significant difference was observed between the 24-h and 48-h results.

The photomicrographs obtained from the scratch wound healing assay are presented in Figures 2 and 3, and the graphical representation of the results are shown in Figure 4. It may be observed that both control and ozone induced the process of wound closure on HaCaT and L929 cell lines. Although no statistical difference between treatment and control was determined, the wound was wider in almost all periods whenever ozone was administered, when compared to control. For HaCaT cells, the

relative wound area treated with ozone varied from 93% (hour 4) to 15% (hour 96), when compared to the initial area at hour zero, and control wound areas varied from 94% (hour 4) to 10% (hour 96). For L929 cells, areas of treated wounds varied between 99% (hour 1) and 41% (hour 12), whereas control areas varied between 100% (hour 1) and 35% (hour 12). The 12-h time point was the last used to calculate the wound area for L929 cell line, due to its densely intertwining growth pattern.

Images of the lower chambers obtained from the transwell migration assay are presented in Figure 5, and the relative counting of migrated cells may be seen in Figure 6. No substantial difference was noticed between control and treatment with ozone for HaCaT cells. In fact, a less intense cell migration was observed for groups treated with ozone (84%). A completely different effect was induced by ozone on the fibroblasts. The migration of L929 cells treated with ozone was of 309%, three times the number of migrated cells counted in the control group ($p<0.05$).

The antimicrobial activity of ozonated PBS, chlorhexidine and their association was assessed. Considering the *S. aureus* results, it may be observed that the association of 8 µg/mL ozone with chlorhexidine demonstrated effectiveness in inhibiting bacteria growth, with the MIC ≤ 0.0009% for chlorhexidine, when compared to chlorhexidine alone (MIC = 0.0078%). Considering the results for *C. albicans*, ozone associated with chlorhexidine was also the most effective treatment, with a MIC of 0.0039%, while chlorhexidine alone resulted in a MIC of 2%. Ozone alone, at a maximum concentration of 8 µg/mL, did not induce a visually perceptible growth inhibition in the evaluated microorganism strains, even though its association with chlorhexidine resulted in antimicrobial activity.

DISCUSSION

In light of the effects described for ozone therapy by experimental and clinical studies, we intended to further evaluate the cytotoxicity effect that ozone yields on keratinocytes and fibroblast and its antimicrobial activity, as well as to assess the impact that it has on the wound healing process. To do so, two cell lines, HaCat and L929, were evaluated with the MTT cell viability assay and cell migration assays, and *S. aureus* and *C. albicans* strains with an antimicrobial susceptibility assay, all after treatment with ozonated phosphate-buffered saline.

Keratinocytes, fibroblast, macrophages and neutrophils are essential cell types for the wound repair, considering their specific roles within this process. For this reason, while managing a post-surgical or an accidental traumatic wound, the option for a therapy that does not impair their functions or reduce their viability is of utter importance. Chlorhexidine is often applied as a pre-surgical skin and mucosa surface disinfectant, as part of intra-operative irrigating solutions and as a wound dressing or post-surgical mouth rinse (*Barnes et al.*, 2014, *Edmiston et al.*, 2015, *Fomete et al.*, 2015, *Vlcek et al.*, 2015). In spite of its well described antimicrobial effects and wide use in treatment of wounds, chlorhexidine toxicity has also been reported to be high. Li et al. (2014) demonstrated that chlorhexidine, at a 0.001% concentration, induced a significant cell viability reduction in macrophages after 1 h of treatment, with rates less than 50%, as well as necrosis and DNA damage at even lower concentrations after 2 h. Faria et al. (2009) reported a considerable induction of apoptosis at concentrations varying between 0.0005% and 0.002% and necrosis at concentrations from 0.002% to 0.016% in L929 cells, possibly as a consequence of endoplasmic reticulum stress. These

observations are in agreement with our results, which showed a significant reduction in L929 and HaCaT cell viability after 24 h and 48 h of treatment with 0.2% chlorhexidine. Such concentration was chosen for our experiments because that is how dentists prescribe it as a post-surgical mouth rinse (*Fomete et al.*, 2015, *Vlcek et al.*, 2015).

While the antimicrobial activity of chlorhexidine and ozone might be comparable (*Montevecchi et al.*, 2013, *Kaur et al.*, 2014), ozone at therapeutic concentrations does not share with chlorhexidine its cytotoxic pattern. Regarding cytotoxicity on cell culture, Costanzo et al. (2015) evaluated ozone at a maximum concentration of 20 µg/mL on HeLa cells (cervical cancer cell line) for 24 h and 48 h, and observed no significant cytotoxicity nor apoptosis for that cell line. Al-Saadi *et al.* (2015) noticed no cell viability reduction on primary osteoblasts after 24 h of a 60-min continuing exposure to ozonated saline solution. Our study also resulted in no cytotoxic effects of ozone at concentrations up to 8 µg/mL to either keratinocytes or fibroblasts, as demonstrated by the MTT assay results.

To evaluate the effect of ozone on cell migration, two methods were used: scratch wound healing assay and transwell migration assay. Both aimed to assess cell migration by mimicking in vivo circumstances and conditions of a wound and its repair. The first method is based on the migration of cells from the wound edges to the central cell-free zone, and the second method on the migration of cells through a porous membrane (*Hulkower and Herber*, 2011). Although no significant difference between control and ozone-treated cells was observed in the scratch assay, a considerable increase in fibroblast migration was noticed on cells treated with 8 µg/mL ozonated saline, when compared to control.

Cell migration is a key event in the wound healing process, and the migration of fibroblasts is especially important during the second stage of wound healing, when the granulation tissue is formed and fibroblasts are recruited by growth factors produced by macrophages (*Zielins et al.*, 2014). Zhang *et al.* (2014) studied the expression of growth factors on biopsy tissues of patients with diabetes foot ulcers treated with ozone therapy, and they detected a significant increased expression of TGF-β, VEGF and platelet-derived growth factor (PDGF) in samples from the treatment group in comparison to control, as well as a greater wound size reduction ($p<0.001$). An increase in fibroblast migration, as the one observed in the transwell assay, might be justified by an overexpression of growth factors and might explain the aiding effect that ozone exerts on wound repair, which has been described in clinical and experimental studies (*Patel et al.*, 2011, *Zhang et al.*, 2014, *Buyuk et al.*, 2015).

As in our study, the effective action of ozonated PBS combined with chlorhexidine on the viability of *C. albicans* yeast has been demonstrated by Noites et al. (2014) and Gopalakrishnan and Parthiban. Even though the inactivation of microorganisms by ozone is considered a complex process, due to the large amount of cell components that can be compromised by its action (*Zhang et al.*, 2011), ozonated solutions represent a promising antimicrobial therapy, especially if used as a co-adjuvant therapy for treating resistant microorganisms infections.

CONCLUSION

Based on the experimental conditions of this study and in accordance with the methodology used, it is possible to confirm the wound healing and antimicrobial potential of ozone therapy. In cell culture assays, it did not show cytotoxic effect on fibroblasts or keratinocytes and it induced fibroblasts migration, which could aid the

wound healing process. Moreover, when used in conjunction with chlorhexidine, it exhibited a greater potential in inhibiting bacteria and yeast growth. Nevertheless, more studies, including animal models studies, are necessary to elucidate the exact molecular mechanisms through which ozone therapy exerts its biological effects.

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Figure 1. Cell viability after 24 h and 48 h, assessed by MTT assay. No statistically significant alteration in cell viability was observed after treatment with ozone. Chlorhexidine reduced cell viability in both cell lines. (A) Cell viability for HaCaT cell line. (B) Cell viability for L929 cell line. Chx, chlorhexidine. *$p<0.05$ *vs.* control (Kruskal-Wallis with Dunn). These results are representative of at least three independent experiments and are presented as median ± range.

Figure 2. Scratch assay with HaCaT cell line. No difference was observed between control and treatment groups. White lines mark the margins of the wound in hour zero in each photomicrograph. (A−E) Control. (F−J) Ozone (8 µg/mL).

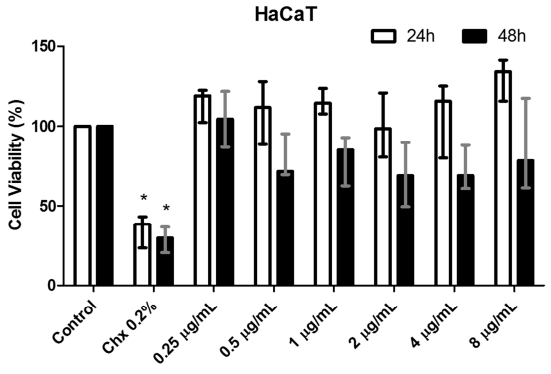
Figure 3. Scratch Assay with L929 cell line. No difference was observed between control and treatment groups. White lines mark the margins of the wound in hour zero in each photomicrograph. (A−E) Control. (F−J) Ozone (8 µg/mL).

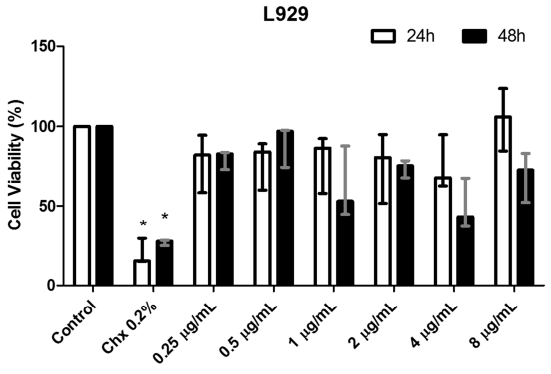
Figure 4. Scratch wound healing assay results. No statistically significant difference between relative areas of control groups and relative areas of treatment groups was observed. Wound area was measured in different periods and compared to the area observed in hour zero. The relative areas observed in each period are presented for both control and treatment with ozone in HaCaT (A) and L929 (B) cell lines. These results are representative of at least three independent experiments and are presented as the mean ± standard deviation (SD).

Figure 5. Transwell migration assay. A visually perceptible difference is observed in the number of migrated L929 cells after treatment with ozone. After a 72-h treatment, cells that migrated from the upper chamber to the lower chamber were stained with Crystal Violet and counted. Photomicrographs represent migrated cells. (A) HaCaT, control. (B) HaCaT, treated with ozone. (C) L929, control. (D) L929, treated with

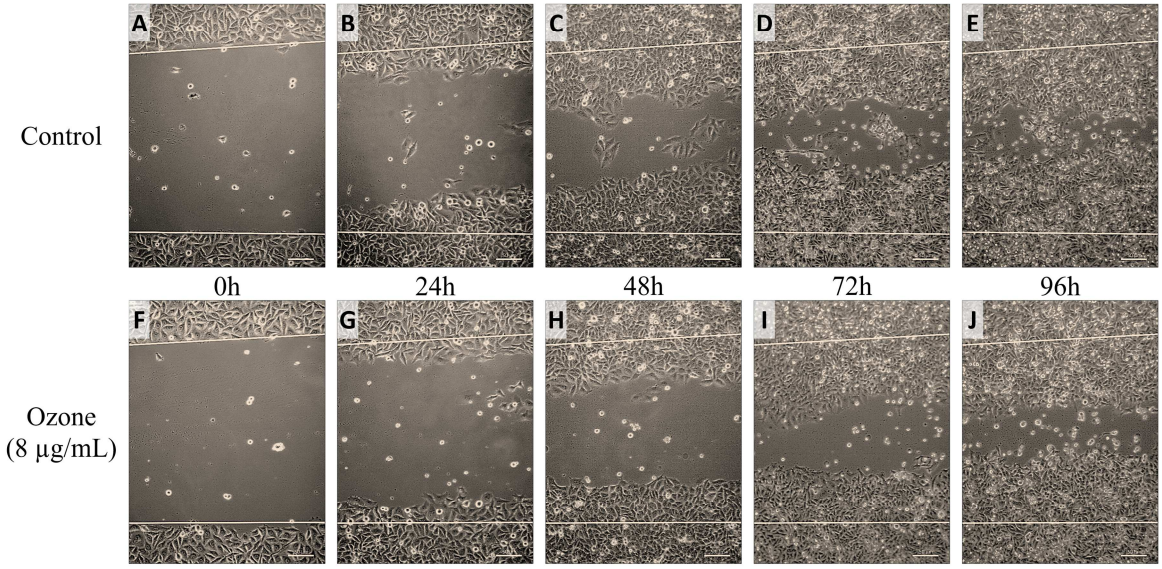
ozone. It is possible to observe that in this treated well, two major groups of cells were formed (#), which are better seen in E and F.

Figure 6. Transwell migration assay. A statistically significant difference in cell counting was found between control and ozone-treated L929 cells. Photomicrographs from lower chambers were taken and cells counted. The relative cell numbers in the lower chambers are presented for HaCaT (A) and L929 (B) cell lines, and were calculated by comparing the number of migrated cells which were treated with ozone to the control. *$p<0.05$ vs. control (Student t-test). These results are representative of at least three independent experiments and are presented as the mean ± SD.

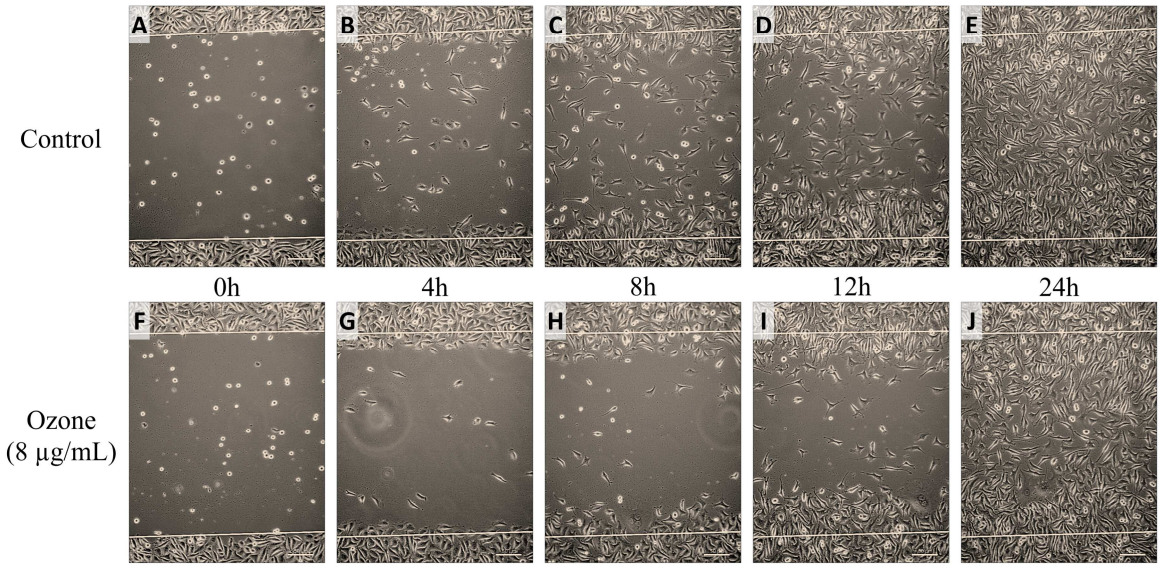


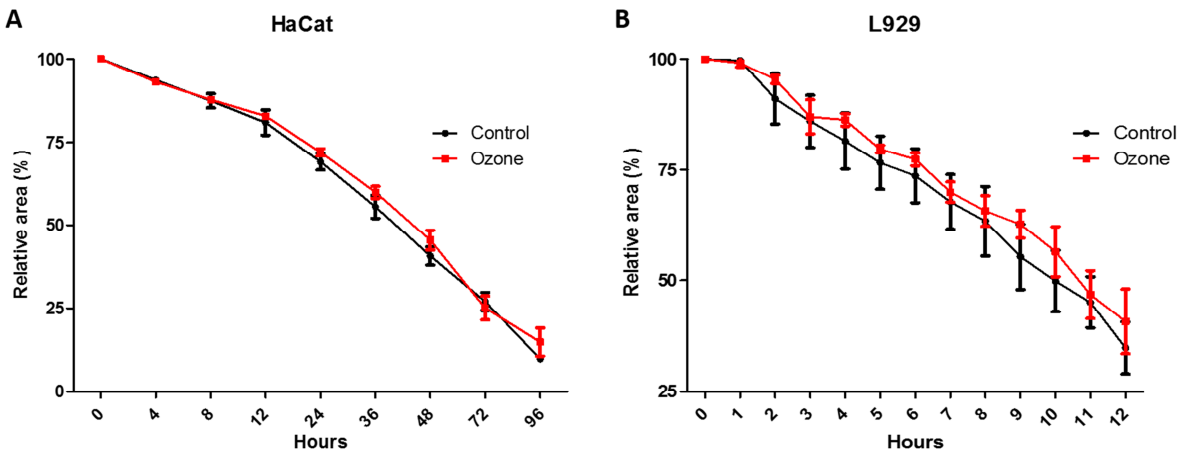


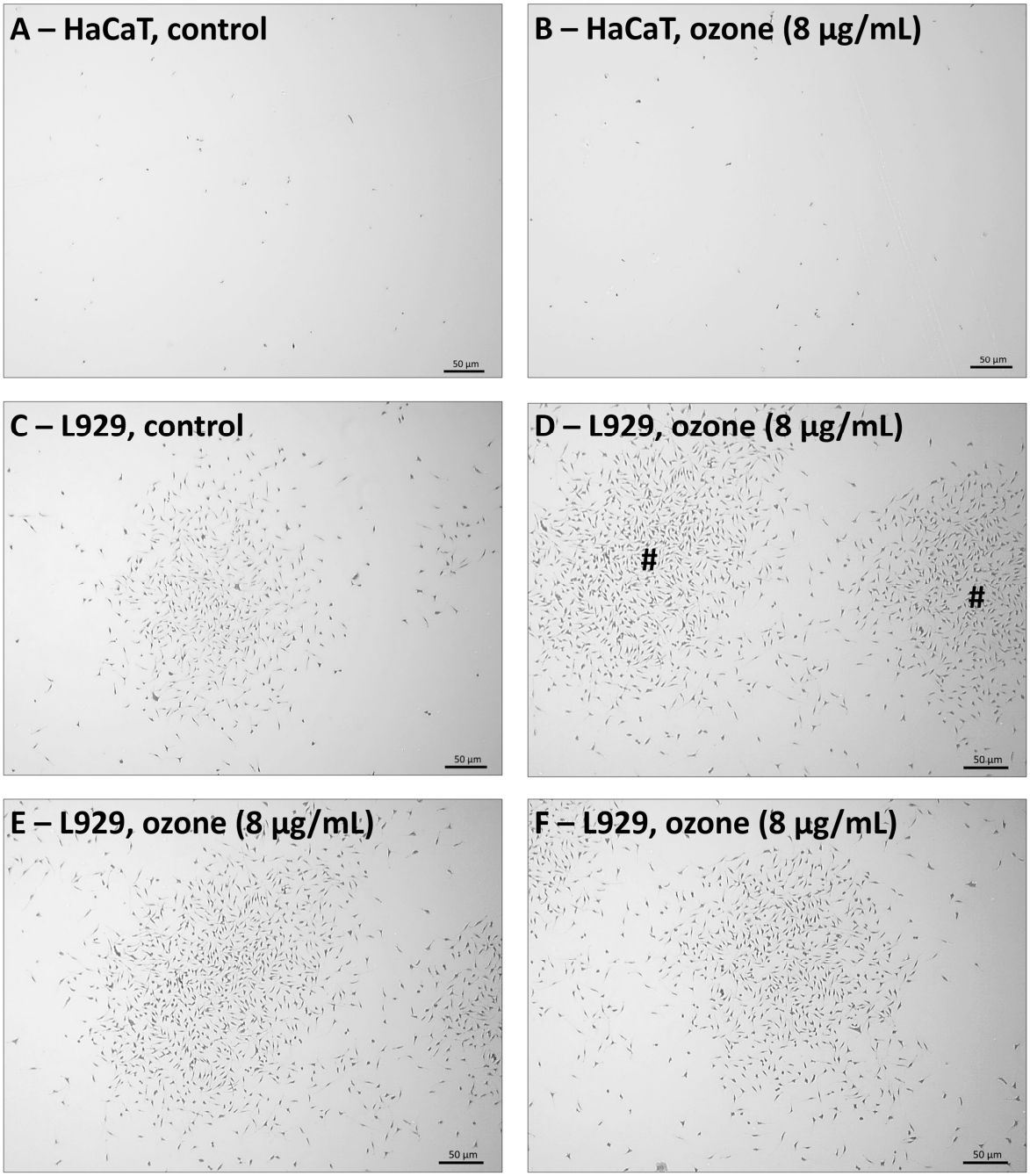
Scratch wound healing assay - HaCaT



Scratch wound healing assay - L929






A – HaCaT, control
B – HaCaT, ozone (8 µg/mL)
C – L929, control
D – L929, ozone (8 µg/mL)
#
#
E – L929, ozone (8 µg/mL)
F – L929, ozone (8 µg/mL)
50 µm