An easy and inexpensive method to expose adhering cultured cells to ozonization

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Summary
The regeneration capabilities of low ozone (O_3) concentrations are usually applied for therapeutic purposes; however, the biological mechanisms accounting for the positive effects of mild ozonization are still largely unknown. Due to the high reactivity of O_3, the study of the cellular effects of ozonization requires controlled and reproducible experimental conditions. An experimental procedure for treating cell suspensions with O_3 has been already set up; however, it is hardly suitable for adhering cells such as epithelial, connective, muscular and neuronal cells. In the present work, we describe a new, easy and cheap method that, without requiring special equipment, proved to be suitable for treating cell monolayers with low O_3 concentrations.

Key words: ozone, *in vitro* culture, epithelial cells.

Introduction

The regeneration capabilities of low ozone (O_3) concentrations are usually applied for therapeutic purposes in a large number of diseases (reviews in Re *et al.*, 2008; Elvis *et al.*, 2011; Bocci, 2012); however, the biological mechanisms accounting for the positive effects of mild ozonization are still largely unknown, being basic research in the field of ozonotherapy scarce (for a scientific overview of O_3 therapy, see ISCO3 2012). Some molecular studies suggested that low ozonization prevents cell damage by enhancing antioxidant pathways (review in Sagai and Bocci, 2011), whereas no data are available on the structural and functional effects of low O_3 concentrations on single cells.

To investigate the cellular effects of ozonization it is essential to ensure controlled and reproducible experimental conditions: to do this, 1) an *in vitro* model is preferable since it allows to exclude the confusing influence of the organismic reaction which inevitably occurs in animal models *in vivo*; 2) gas flow rate and O_3 concentration need to be exactly determined; 3) due to the high O_3 reactivity, rapid and precise handling is necessary; 4) gas administration must take place maintaining the pressure at the normal atmospheric level.

An excellent experimental procedure for treating cell suspensions with O_3 was set up by Larini *et al.* (2003), but it is hardly suitable for adhering cells such as epithelial, connective, muscular and neuronal cells. It is obviously possible to treat these cells in suspension and then allow them to adhere, but this represents a strong perturbation of their physiological condition and may cause artifactual alterations, especially when fine analyses of cell structural components have to be performed. With the aim to investigate the effects of mild ozonization on epithelial cells focussing on the structure and function of some cell organelles, we modified the well-established procedure by Larini *et al.* (2003), setting up an easy and cheap method for treating cell monolayers with low O_3 concentrations under strictly controlled experimental conditions.

Materials and Methods

Cell culture and gas treatment

HeLa cells were grown in DMEM (Dulbecco Modified Eagles Medium) supplemented with 10% (v/v) fetal calf serum, 1% (w/v) glutamine, 100 U of penicillin and 100 µg/mL streptomycin (Celbio, Milan, Italy), at 37°C in a 5% CO_2 humidified atmosphere. An appropriate number of cells (2.5×10^4) were planted onto glass coverslips (24×24 mm) placed on the wells' bottom in 6 multiwell plastic dishes (Corning Inc., Corning, NY, USA), and treated 24 hours post-seeding.

The different phases of the method are shown in Figure 1. The coverslips were located into a 50 ml polypropylene (O_3 resistant) syringe (Terumo Medical Corporation, Somerset, NJ, USA) containing 20 mL of culture medium. In order to hold up and protect the coverslips during the treatment (which could break them and/or damage the cell monolayer), a piece (25 × 25 × 6.4 mm) of a glass strip for making glass knives for resin sectioning (Electron Microscopy Sciences, Hatfield, PA, USA) was placed inside the syringe; this allowed to treat two coverslips at the same time. The syringe was connected to a tube from a blood transfusion set (suitable for O_3 therapy) (Aries srl, Mirandola, Italy). The tube length was shortened, by cutting with a sterilized razor blade, to the minimum necessary for handling: 3 cm from the syringe tip to the flow regulator (a portion filled with the culture medium) and 6 cm from the flow regulator to the male conical fitting (a portion containing air).

An OZO2 Futura apparatus (Alnitec s.r.l., Cremosano, CR, Italy) which generates O_3 from medical-grade O_2 and allows photometric real-time control of the gas flow rate and O_3 concentration was used to produce O_2 and 10 µg/mL O_3. The syringe containing the coverslips dipped in 20 mL of culture medium was connected by the tube to the OZO2 Futura output valve and an equal volume of gas (O_2 or 10 µg/mL O_3) was collected through a sterile filter (Alnitec s.r.l.) in order to avoid contamination.

For air-treated samples, an equal volume of air was aseptically collected in the laminar flow hood.

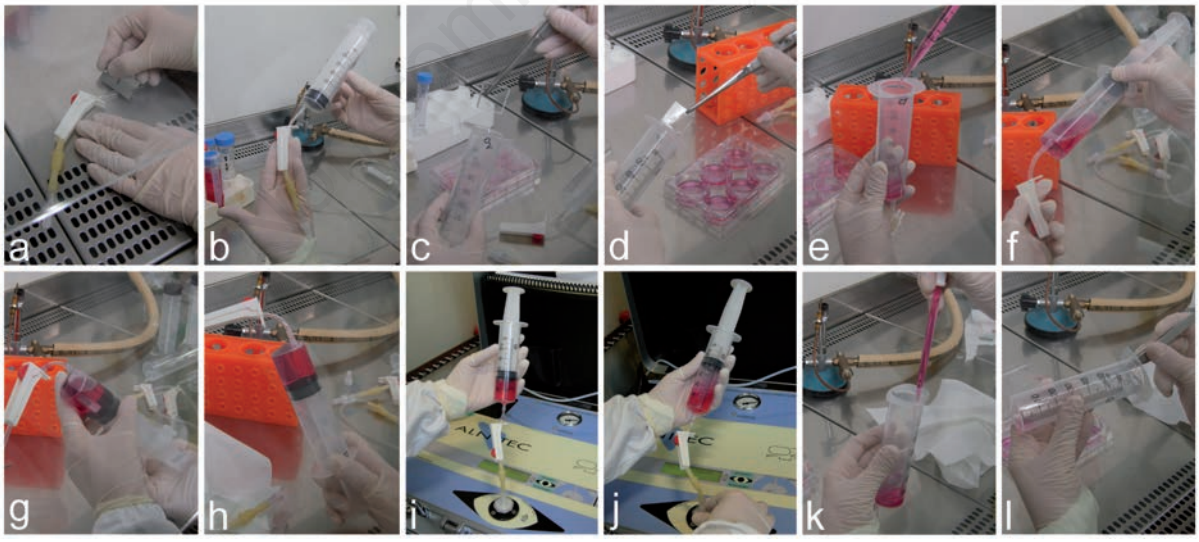

Figure 1. Illustration of the experimental procedure. The tube is cut at the appropriate length (a) and is connected to the syringe tip (b). After removing the plunger, the piece of glass is placed inside the syringe (c) and then the coverslips are located at its two sides (d). The culture medium is gently poured into the syringe (e); then, keeping open the flow regulator, the plunger is introduced (f) and pushed (g) to exclude the air from the syringe (h). After closing the flow regulator, the tube tip is connected to the output valve of the ozone generator by a sterile filter (i). The flow regulator is open, to allow the gas to enter the syringe (j). The flow regulator is closed again and, after 10 minutes, the syringe plunger is removed and the culture medium extracted (k). Finally, the coverslips are removed (l).

After collecting the gas, the syringe was placed in the laminar flow hood and gently moved for 10 min to dissolve the gas in the medium. Then, the coverslips were taken out of the syringe and re-placed in the wells containing fresh culture medium.

As control, cells adhering to coverslips were kept in the well, and then analysed together with treated samples.

All samples were analysed for cell viability, cell morphology and oxidative stress at different times post-treatment (see below).

Cell viability

To determine the effect of gas exposure on cell survival, control and treated cells (1h, 6h, 24h and 48h after O_3 exposure) were stained for 2 min with 0.1% trypan blue in the culture medium: cells that were permeable to the dye were considered as non-viable and their percentage was estimated by counting on 20 randomly chosen optical fields at 40x magnification in an Olympus BX51 microscope (Olympus Italia Srl, Milan, Italy).

Data were expressed as the mean of three independent experiments ± standard error (SE). Statistical comparisons were performed by the one way-Anova test.

Cell morphology

To evaluate general cell morphology, 24h after treatment the samples were fixed with 4% (v/v) formaldehyde (30 min at room temperature), then with 70% ethanol in water (30 min at -20°C), hydrated with PBS and stained with 0.1% toluidine blue in water for 1 min, rinsed in PBS, and finally mounted in a 1:1 PBS:glycerol mixture.

To visualize the cytoskeletal microfilaments, after treatment the cells were fixed with 4% (v/v) formaldehyde (30 min at room temperature) and 70% (v/v) ethanol in water (30 min at -20°C), rehydrated with PBS, incubated with Alexa 488-conjugated phalloidin (Molecular Probes, Invitrogen, Italy) diluted 1:40 in PBS for 1 h at room temperature, stained for DNA with Hoechst 33342 (0.1 µg/mL in PBS for 10 min), rinsed in PBS, and finally mounted in 1:1 PBS:glycerol. These samples were also used to evaluate the percentage of mitotic and apoptotic cells (identified by chromatin morphology) as above described for cell viability. For observation of all samples, an Olympus BX51 microscope was used; for fluorescence microscopy, a 100W mercury lamp was used under the following conditions: 450-480 nm excitation filter (excf), 500 nm dichroic mirror (dm), and 515 nm barrier filter (bf) for Alexa 488; 330–385 nm excf, 400-nm dm, and 420 nm bf, for Hoechst 33258. Images were recorded with a QICAM Fast 1394 Digital Camera (QImaging, Surrey, BC, Canada) and processed with Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA).

Detection *in situ* of Reactive Oxygen Species (ROS)

The intracellular sites of ROS (superoxide anion radical and singlet oxygen) production were visualised by the cytochemical method based on a diaminobenzidine (DAB)-Mn^{2+}-Co^{2+} reaction (Freitas *et al.*, 2002). Briefly, 30 min and 5h post-treatment HeLa cells were incubated for 30 min in a medium containing 12.5 mM DAB, 5 mM $MnCl_2$ and 40 mM $CoCl_2$ dissolved in 10% w/v polyvinyl alcohol, in 100 mM Tris-maleate buffer pH 8.0 at 37°C. The cells were then extensively rinsed in hot (60°C) distilled water and finally mounted in PBS:glycerol (1:1). As a positive control of the cytochemical reaction, HeLa cells treated with 30% H_2O_2 for 2h at 37°C were treated as above. The samples were observed in differential interference contrast (DIC) using an Olympus BX51 microscope.

Results and Discussion

Maintaining cells in a condition as close as possible to the physiological state is essential to evaluate properly the effects of any experimental treatments. In the present study, we verified the reliability of an original experimental procedure to expose cell monolayers to gaseous treatment. In particular, we were interested in setting up a system to expose cell monolayers to O_3/O_2 gas mixtures, in the frame of a research aimed at investigating the cellular mechanisms responsible for the therapeutic effects of mild ozonization.

We demonstrated that gas (10 µg/mL O_3, O_2 and air) treatment in the syringe does not induce cell death; in fact, trypan blue-positive cells were lower than 1% in all samples for any post-treatment time considered, with no significant difference with control samples (i.e., untreated cells maintained in the well). Accordingly, similar results were found for the apoptotic cell percentage, which was negligible, ranging from 0.10±0.04

to 0.12±0.07% in all samples. The treatment did not affect the mitotic index too, that was found to range from 0.34±0.11 to 0.53±0.12%, without significant difference among samples.

The general morphology, investigated by toluidine blue staining (Figure 2), demonstrated that nor gas flow nor handling did injure the cell monolayer. The cells were firmly adherent and flattened, and uniformly distributed in all the samples treated in the syringe, similarly to control cells maintained in the well.

This observation was confirmed by fluorescent phalloidin labelling of actin (Figure 3), which showed that, 24h post-treatment, the arrangement of the cytoskeletal microfilaments was not altered by gas exposure or mechanical treatment. In addition, phalloidin labelling revealed that cells treated with 10 µg O_3/mL were more distended than those of all the other samples (control included), according to our previous observations (manuscript in preparation) as well as to literature data suggesting a dose-dependent effect of ozone on actin polymerization (Taulet *et al.* 2012; Muliyil and Narasimha, 2014).

The method based on a DAB-Mn^{2+}-Co^{2+} reaction demonstrated that positive cells were less than 0.1% in all samples both 30 min (Figure 4) and 5h (not shown) post-treatment, thus excluding oxidative damage for all samples treated in the syringe. The reliability of this cytochemical method (Freitas *et al.*, 2002) was confirmed by the positive control after H_2O_2 treatment, where cells showed diffuse and granular blue staining in both the cytoplasm and nucleus.

Taken together, these results demonstrate that our experimental procedure allows treatment of cell monolayers with gas flow produced by the ozone generator without inducing evident cell

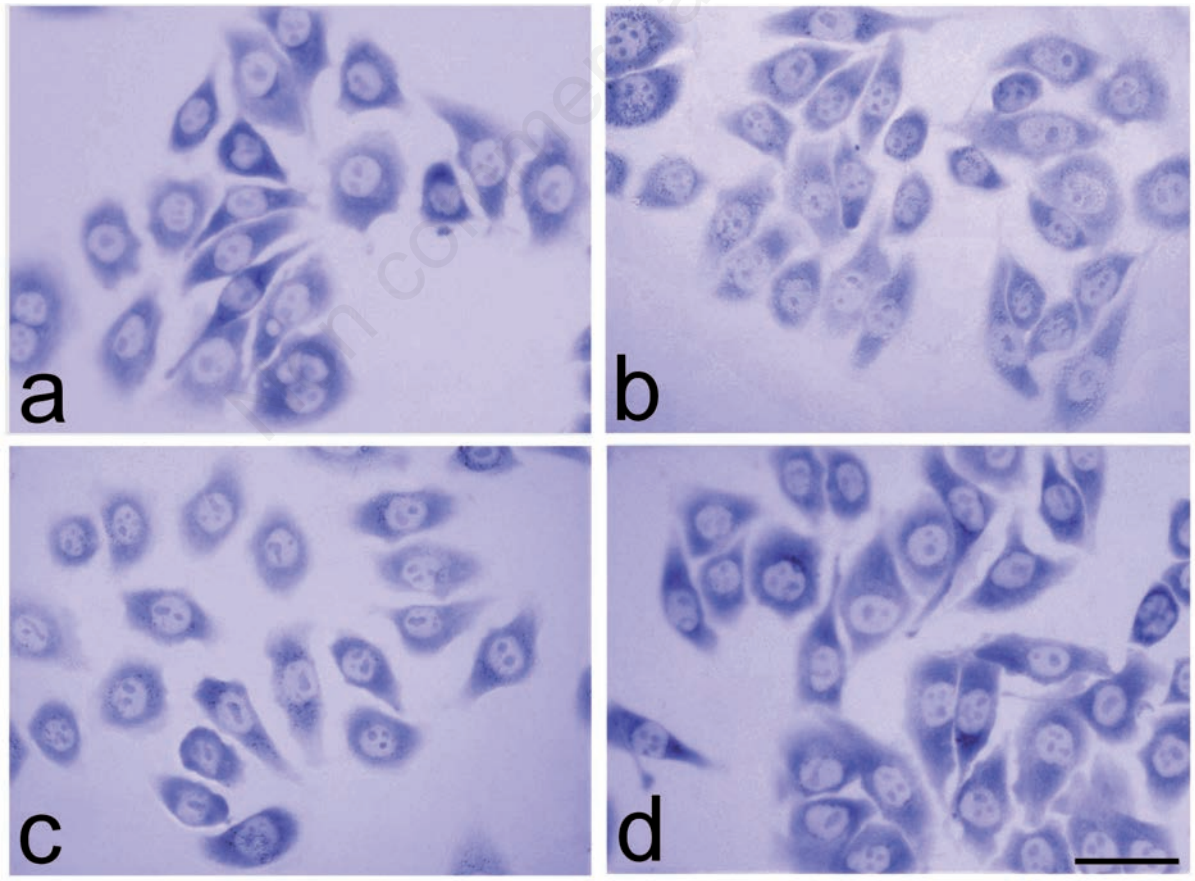

Figure 2. HeLa cell monolayers from control (a), air-treated (b), O_2-treated (c) and 10 µg/mL O_3-treated (d) samples. Toluidine blue staining. In all samples, cell morphology is well preserved. Bar: 40 µm.

damage or alteration.

By our system, the gas pressure inside the syringe is maintained at the atmospheric one. In addition, the contact between gas and culture medium (and consequently the cells) takes place immediately after emission from the generator, and no additional passages are needed. This is essential to ensure controlled experimental conditions, being O_3 an extremely reactive gas.

It should be underlined that the short portion of the blood transfusion tube with its flow regulator is necessary for preventing culture medium from dropping and dampening the filter, thus hampering gas flow. However, the volume of the air contained in the tube can be considered as negligible since it corresponds to about 0.15% of the total amount of gas present in the syringe.

In conclusion, the technique we propose does not require special devices or equipment to be performed; it is repeatable, easy and cheap, and it allows to expose cultured cell monolayers to the ozone treatment. This is extremely convenient especially for investigating the effects of ozonization at short times post-treatment, which is prevented when adhering cells are exposed to ozone in suspension and relatively long times (sometimes hours) are needed for cells to adhere again to the growing substrate. In particular, this method promises to be suitable for studying the effects of ozone on the molecular mechanisms of cell adhesion or on the cytoskeleton-related transport of vesicles during endo- and exocytosis.

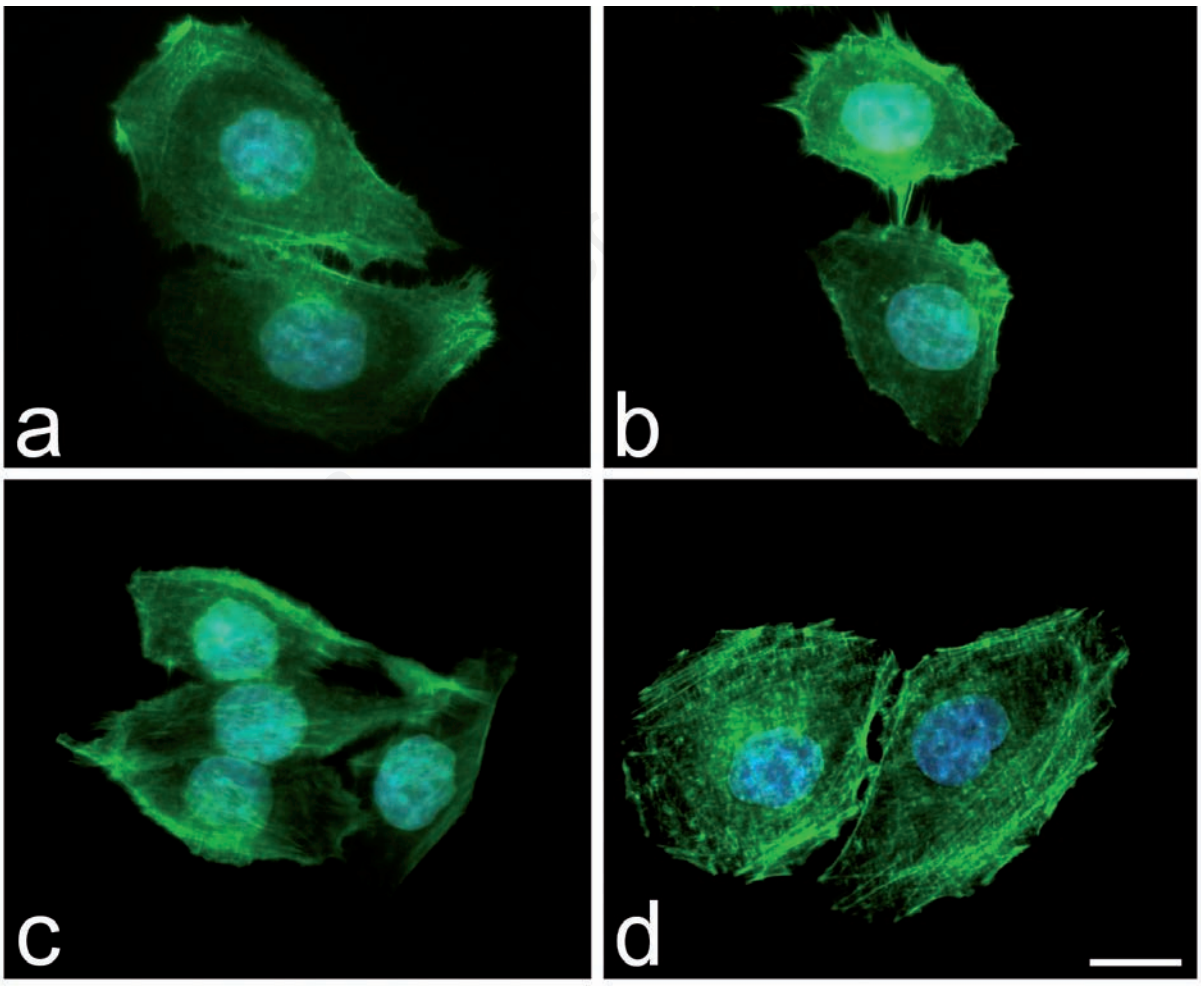

Figure 3. Fluorescence micrographs of HeLa cells from control (a), air-treated (b), O_2-treated (c) and 10 µg/mL O_3-treated (d) samples. Cytoskeletal actin was labelled with Alexa 488-conjugated phalloidin (green); nuclear DNA was stained with Hoechst 33342 (blue). The actin filaments of 10 µg/mL O_3-treated cells are more evident that in the other samples. Bar: 20 µm.

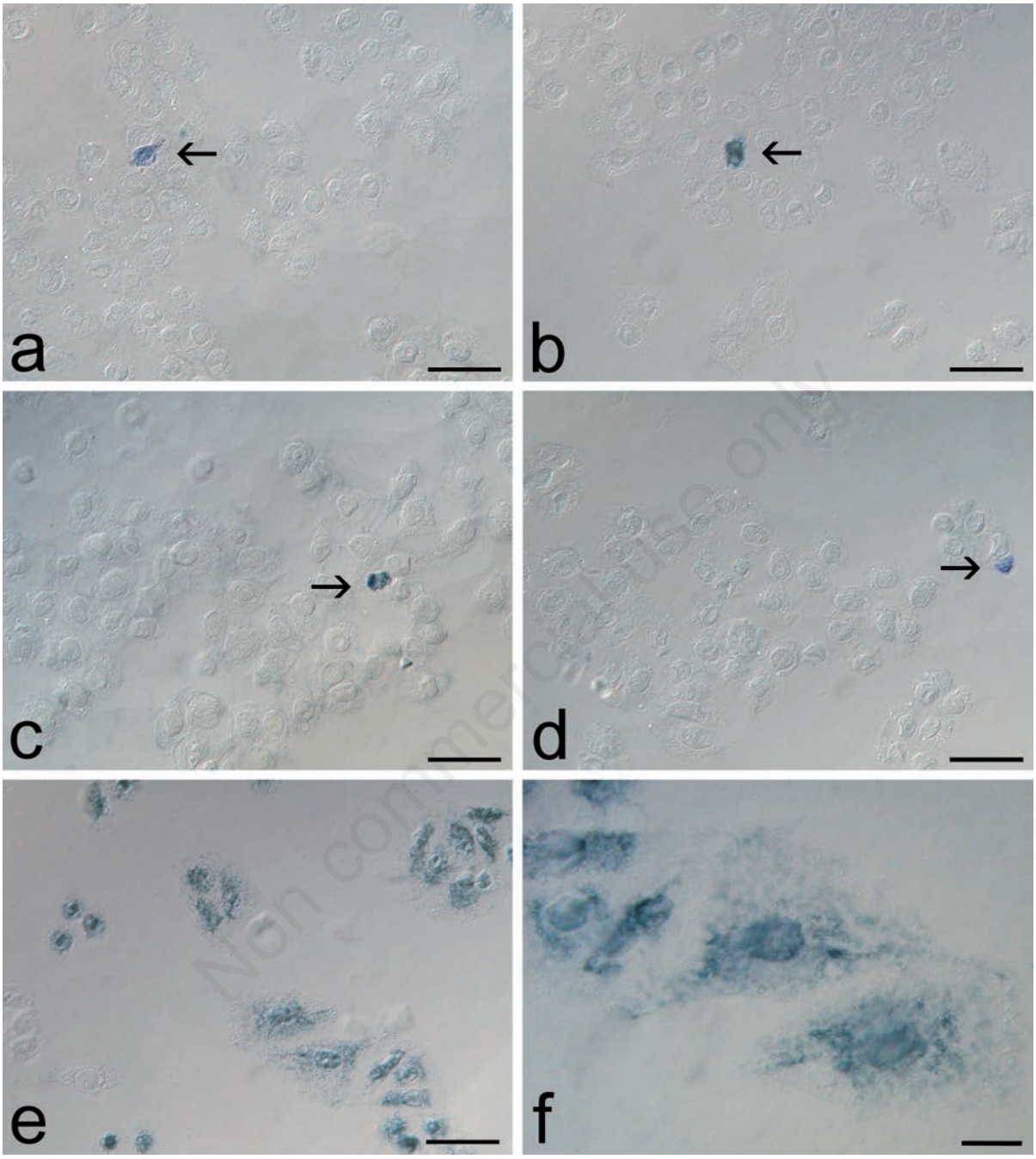

Figure 4. Differential interference contrast (DIC) micrographs of HeLa cell monolayers from control (a), air-treated (b), O_2-treated (c) and 10 µg/mL O_3-treated (d) samples. DAB-Mn^{2+}-Co^{2+} reaction for detection of ROS. Note the very low number of cells showing intracellular blue precipitates (arrows) in all samples. e,f) H_2O_2-treated cells (positive control of the cytochemical reaction). The number of positive cells is high (e) and the blue final reaction product occurs in both the cytoplasm and nucleus (f). Bars: a-e, 50 µm; f, 20 µm.

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