Hypochlorous acid selectively promotes toxicity and the expression of danger signals in human abdominal cancer cells

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Abstract. Tumors of the abdominal cavity, such as colorectal, pancreatic and ovarian cancer, frequently metastasize into the peritoneum. Large numbers of metastatic nodules hinder curative surgical resection, necessitating lavage with hyperthermic intraperitoneal chemotherapy (HIPEC). However, HIPEC not only causes severe side effects but also has limited therapeutic efficacy in various instances. At the same time, the age of immunotherapies such as biological agents, checkpoint- inhibitors or immune-cell therapies, increasingly emphasizes the critical role of anticancer immunity in targeting malignancies. The present study investigated the ability of three types of long-lived reactive species (oxidants) to inactivate cancer cells and potentially complement current HIPEC regimens, as well as to increase tumor cell expression of danger signals that stimulate innate immunity. The human abdominal cancer cell lines HT-29, Panc-01 and SK-OV-3 were exposed to different concentrations of hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$). Metabolic activity was measured, as well as determination of cell death and danger signal expression levels via flow cytometry and detection of intracellular oxidation via high-content microscopy. Oxidation of tumor decreased intracellular levels of the antioxidant glutathione and induced oxidation in mitochondria, accompanied by a decrease in metabolic activity and an increase in regulated cell death. At similar concentrations, HOCl showed the most potent effects. Non-malignant HaCaT keratinocytes were less affected, suggesting the approach to be selective to some extent. Pro-immunogenic danger molecules were investigated by assessing the expression levels of calreticulin (CRT), and heat-shock protein (HSP)70 and HSP90. CRT expression was greatest following HOCl and $ONOO^-$ treatment, whereas HOCl and H_2O_2 resulted in the greatest increase in HSP70 and HSP90 expression levels. These results suggested that HOCl may be a promising agent to complement current HIPEC regimens targeting peritoneal carcinomatosis.

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Introduction

Several types of tumor can metastasize on or into the peritoneum (1). Rates of <30% have been reported (2), making peritoneal carcinomatosis (PC) a burden for patients and healthcare systems alike. Pancreatic carcinoma often is diagnosed late and shows aggressive growth and metastasization in the peritoneum, limiting effective therapies (3,4). Similarly, colorectal and ovarian carcinoma display widespread localization in the abdominal cavity, which allows open field metastasization and generation of PC before diagnosis (5-7). Curative treatment is rarely achievable. For palliation, systemic chemotherapy is of low efficacy and causes strong side effects (8). The combination of cytoreductive surgery with hyperthermic intraperitoneal chemotherapy (HIPEC; peritoneal lavage with heated liquids containing chemotherapeutic agents) has similar limitations (9). For HIPEC, life expectancy and quality of life in patients with PC are low (10). Alternative treatments, such as laser-induced oxidation of peritoneal cancer, may prolong survival but have been shown to be impractical and cause severe side effects (11).

Reactive oxygen species (ROS) are increasingly recognized as critical agents in anticancer therapy (12-15). For instance, certain types of nanoparticle (such as metal oxides, carbon nanotubes and silver nanoparticles) promote stress of the endoplasmic reticulum and mitochondria via ROS (16-18). Certain chemotherapeutic agents, such as 5-fluorouracil, can lead to generation of intracellular oxidants, such as peroxynitrite ($ONOO^-$), in tumor cells (19). Anthracyclines also generate intracellular ROS (20). Some treatment strategies aim to directly generate ROS. These include photodynamic therapy (PDT), which locally generates singlet Δ oxygen via a photosensitizer (21). PDT also promotes pro-immunogenic properties in tumor cells (22). Similarly, gas plasma treatment generates a multiple types of reactive oxygen and nitrogen

species (ROS/RNS) simultaneously (23-25). This is not only toxic to tumor cells but also promotes their immunogenicity via an increase in danger signals (26-28). Specific pattern recognition receptors (PRR) are sensed by evolutionary preserved structures, such as pathogen- and damage-associated molecular patterns [including ATP, heat-shock proteins, endoplasmic reticulum chaperon calreticulin (CRT)]. Innate immune cells are thus able to initiate quick and early responses against infection or extensive cell death in tissue. These mechanisms emphasize the important role of immunogenic cell death (ICD) in initiating an anti-tumor immune response (29-31).

Since the award of the Nobel Prize for Physiology or Medicine in 2018 for immune-checkpoint therapies, there has been an increasing awareness of combination of conventional treatment modalities with immunotherapy (32). For PC treatment, standard therapy includes the intraperitoneal administration of chemotherapeutics that directly reach tumor lesions but cannot stimulate immunity via danger signals derived from targeted cancer cells (33,34). This limitation may be overcome by using liquids supplemented with ROS to not only generate cytotoxic responses in tumor cells but also render them more immunogenic by promoting the expression of danger signals (35). To this end, the present study compared three types of ROS, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and $ONOO^-$, to investigate their ability to inactivate cancer cells and promote expression of markers associated with ICD (31).

Materials and methods

Cell culture. A total of three human abdominal cancer cell lines (HT-29 colorectal, Panc-01 pancreatic and SK-OV-3 ovarian cancer cells), as well as non-malignant HaCaT keratinocytes, were used. The HT-29 and Panc-01 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.), while SK-OV-3 and HaCaT cells were cultured in RPMI-1640 medium (PAN Biotech GmbH). Both media were supplemented with 10% fetal bovine serum, 2% penicillin-streptomycin and 1% glutamine (all Sigma-Aldrich; Merck KGaA). Cells were cultured at 37˚C, 5% CO_2 and 95% humidity in a cell culture incubator (Binder GmbH). Subculturing was performed 2-3 times/week. In order to determine the cell count for downstream experiments at high precision, cells were stained with PI (Sigma-Aldrich; Merck KGaA) for 5 min at room temperature, and absolute counts were obtained using acoustic focusing flow cytometry (Attune Nxt; Thermo Fisher Scientific, Inc.) and Attune Nxt Software v. 2.7.0 (Thermo Fisher Scientific, Inc.). Cells were seeded at $1x10^5$ cells in flat-bottom 24-well plates for flow cytometry or $1x10^4$ cells in 96-well plates (both Eppendorf) for imaging experiments. The outer rim of each plate was filled with double-distilled water to prevent surplus evaporation in the edge wells.

Oxidant treatment. H_2O_2 (Alfa Aesar; Thermo Fisher Scientific, Inc.), HOCl and $ONOO^-$ (both Carl Roth GmbH & Co.) were diluted in PBS to obtain concentrations between 10 mM and 0.1 μM. Cell culture medium was removed and 250 μl (per $1x10^5$ cells in 24-well plates) or 25 μl (per $1x10^4$ cells in 96-well plates) individual oxidant-containing liquid was added to cells. After 2 h of incubation at 37˚C, 1,750 (for 24-well plates) or 175 μl culture medium (for 96-well plates) was added to the cells to restore preferred culture conditions with fully supplied nutrients. The cells were allowed to culture for 22 h prior to further downstream processing.

Metabolic activity. The total metabolic activity per well was analyzed based on cell capability to metabolize 7-hydroxy-3 H-phenoxazin-3-on-10-oxid (resazurin; Alfa Aesar; Thermo Fisher Scientific, Inc.) to fluorescent resorufin. Resazurin was added to wells at a final concentration of 100 μM and incubated for 2 h at 37˚C before fluorescence was quantified using a multimode plate reader (F200; Tecan Group Ltd.). The measurement was taken at λ_{ex} 535 and λ_{em} 590 nm.

Flow cytometry. Flow cytometry was performed to investigate cell death and analyze surface marker expression levels. For the collection of cells, cell culture supernatant and detached cells (obtained using accutase; BioLegend, Inc.) were transferred to v-bottom 96-well plates (Eppendorf) after centrifugation at 500 x g for 5 min at room temperature. The plate was washed with PBS (PAN Biotech GmbH) before one of the following mixes was added to the wells: i) DAPI (BioLegend, Inc.) and active caspase-3/7 detection reagent (Thermo Fisher Scientific, Inc.); or ii) DAPI, anti-CRT monoclonal antibodies conjugated with phycoerythrin (Enzo Life Sciences, Inc.; cat. no. ADI-SPA-601PE-F), anti-heat-shock protein (HSP)70 monoclonal antibodies conjugated with Alexa Fluor® (AF) 594 and anti-HSP90 monoclonal antibodies conjugated with AF 700 (both Novus Biologicals LLC; cat. nos. NBP1-77456AF594 and NB100-1972AF700, respectively). The staining was performed for 30 min at 37˚C using 20 ng antibodies per test and DAPI and caspase-3/7 reagent at a final concentration of 1 μM. Subsequently, the cells were washed twice and analyzed using a CytoFLEX S (Beckman Coulter, Inc.) four-laser flow cytometer. The excitation wavelength and bandpass filters for collecting fluorescence emission were λex 405 nm/λ_{em} 450-45 nm and λ_{ex} 488 nm/λ_{em} 520-40 nm, respectively, for master mix i); and λ_{ex} 405 nm/λ_{em} 450-45 nm, λ_{ex} 561 nm/λ_{em} 585-42 nm, λ_{ex} 561 nm/λ_{em} 610-20 nm and λ_{ex} 638 nm/λ_{em} 712-25 nm for master mix ii). Forward and side-scatter were also analyzed. Gating and quantification of cell numbers and fluorescence intensities was performed using Kaluza 2.1.1 analysis software (Beckman Coulter, Inc.).

Quantitative high-content imaging. In order to determine cytosolic glutathione (GSH) levels and mitochondrial redox status, cells were stained with 2 μM GSH detection probe (Kerafast, Inc.) or 1 μM mitotracker orange (MTO; Thermo Fisher Scientific, Inc.) for 90 min at 37˚C. Following exposure to oxidants, as aforementioned, cells were imaged using a high content imaging device (Operetta CLS; PerkinElmer, Inc.). The digital phase contrast (DPC) channel (pseudo-cytosolic signal), as well as the fluorescence channels for bound (λ_{ex} 390-420/λ_{em} 500-550 nm) or unbound GSH tracer or MTO (λ_{ex} 460-790/λ_{em} 570-650 nm), were imaged. The experimental setup, as well as the software-based quantification algorithms, were generated using Harmony high-content imaging and analysis software 4.9 (PerkinElmer, Inc.). For segmentation, cells were detected via DPC signal and the fluorescence of both GSH tracer or MTO channels was quantified. For algorithm-driven quantification, ≥$1x10^4$ individual cells in

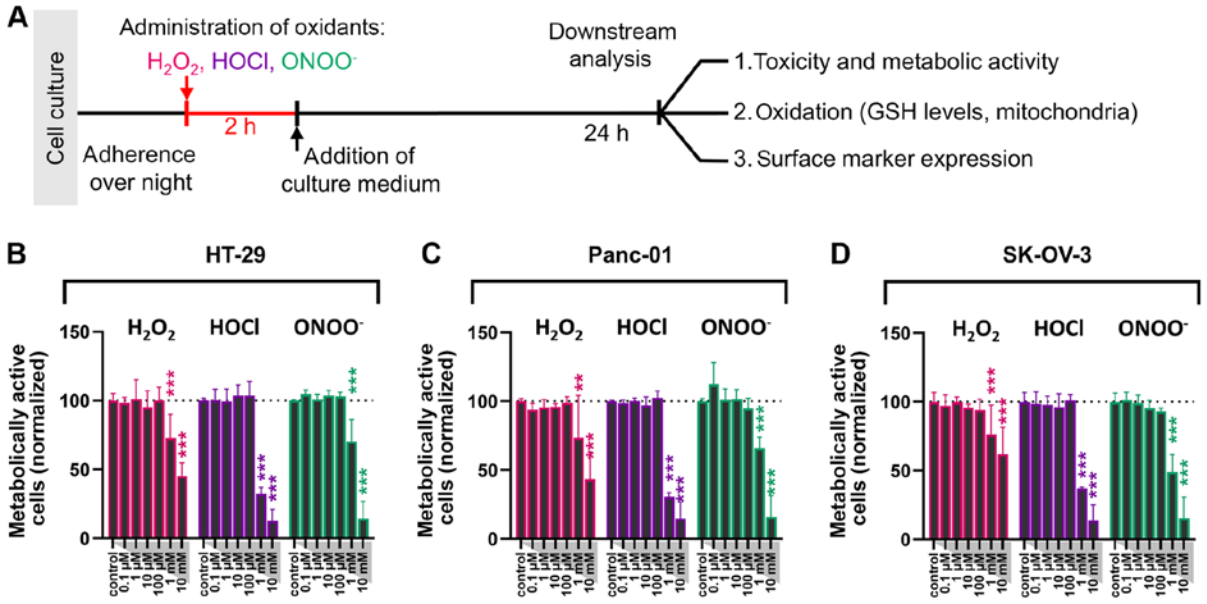

Figure 1. Experimental design and metabolic activity of tumor cells following exposure to oxidants *in vitro*. (A) Experimental design showing the administration of H_2O_2, HOCl or ONOO$^-$ to cancer cells. Percentage of metabolically active (B) HT-29, (C) Panc-01 and (D) SK-OV-3 tumor cells normalized to untreated controls at 24 h post-administration of oxidants. Data are from two to three independent experiments with several replicates each and are presented as the mean ± SD. **P<0.01, ***P<0.001 vs. control. H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; ONOO$^-$, peroxynitrite; GSH, glutathione.

50 single images were quantified per treatment condition. The intracellular GSH levels were calculated according to the formula: GSH level=mean fluorescence intensity (MFI) of bound GSH tracer/MFI of unbound GSH tracer.

Graphing and statistical analysis. Statistical analysis and graphing was performed using Prism 8.4 (GraphPad Software, Inc.). For statistical comparison between groups and controls, two-way analysis of variance with Dunnett's post hoc test was used. All experiments were performed ≥2 times. P<0.05 was considered to indicate a statistically significant difference.

Results

ROS decreases metabolic activity and viability in a dose-dependent manner. The present study aimed to identify the effect of different types of ROS on the viability and immunogenic properties of abdominal cancer cells. Colorectal (HT-29), pancreatic (Panc-01) and ovarian (SK-OV-3) cancer cells were exposed to either H_2O_2, HOCl or ONOO$^-$ at different doses. Following 2 h treatment with these ROS, cells were cultured and analyzed 24 h post-treatment (Fig. 1A). Compared with untreated control cells, oxidant concentrations between 0.1 μM and 100 μM did not significantly decrease cancer cell metabolic activity (Fig. 1B-D). At concentrations >100 μM, H_2O_2 showed the lowest capability in decreasing the percentage of metabolically active tumor cells in the three cancer cell lines tested. HOCl exhibited the most substantial effects (Fig. 1B-D).

In order to analyze the cause of decreased metabolic activity, flow cytometry was used to quantify viable (DAPI$^-$, active caspase$^-$), early (DAPI$^-$, active caspase$^+$) and late apoptotic (DAPI$^+$, active caspase$^+$), and necrotic (DAPI$^+$, active caspase$^-$) tumor cells. A dose-dependent decrease in the number of viable cells was observed in all cell lines (Fig. 2A-C). In HT-29 cells, a significant decrease in the cell viability was found at concentrations ≥10 μM for HOCl and ONOO$^-$, and ≥100 μM for H_2O_2 (Fig. 2A). In Panc-01 cells, a significant decrease in the number of viable cells was observed at ≥10 μM ONOO$^-$, ≥100 μM for HOCl and ≥1 mM for H_2O_2 (Fig. 2B). Similar effects were also observed in SK-OV-3 cells; the number of viable cells significantly decreased at concentrations ≥1 mM H_2O_2 and ONOO$^-$, and 10 μM HOCl (Fig. 2C). Analysis of early and late apoptotic and necrotic cells (Fig. 2D, H and L) demonstrated the greatest extent of cell death occurred in HT-29 cells treated with HOCl (Fig. 2E-G). The fraction of necrotic cells remained small in all groups, except for HOCl-treated HT-29 and SK-OV-3 cells, and ONOO$^-$-treated Panc-01 cells at higher concentrations (Fig. 2F, N and K). In most experimental conditions, the fraction of late apoptotic cells was the largest of the dead cell populations. All oxidants induced notable toxicity in Panc-01 cells at high concentrations, while concentrations ≤10 μM did not have a significant effect (Fig. 2I-K). HOCl-treated cells (10 mM) were not detectable (data not shown). SK-OV-3 cells exhibited notably elevated apoptotic cell death in all oxidative regimens (except for 1 mM HOCl with more necrotic cells); however, ONOO$^-$ at concentrations ≥1 mM failed to generate single-cell data because of aberrant toxicity (Fig. 2M-O).

ROS treatment affects metabolic activity and viability to a lesser extent in HaCaT cells than in cancer cells. In order to investigate the effect of oxidants in non-malignant cells, HaCaT keratinocytes were used as a control cell line. Similar to the tumor cells, a decrease in metabolic activity was observed (Fig. 3A). However, this was only notable at 1 mM. Moreover, viability data from flow cytometry suggested these cells to be less sensitive to oxidative treatment compared with cancer cells; a significant decrease in the number of viable cells was observed only at concentrations >1 mM, except for HOCl, which also exhibited a significant effect at

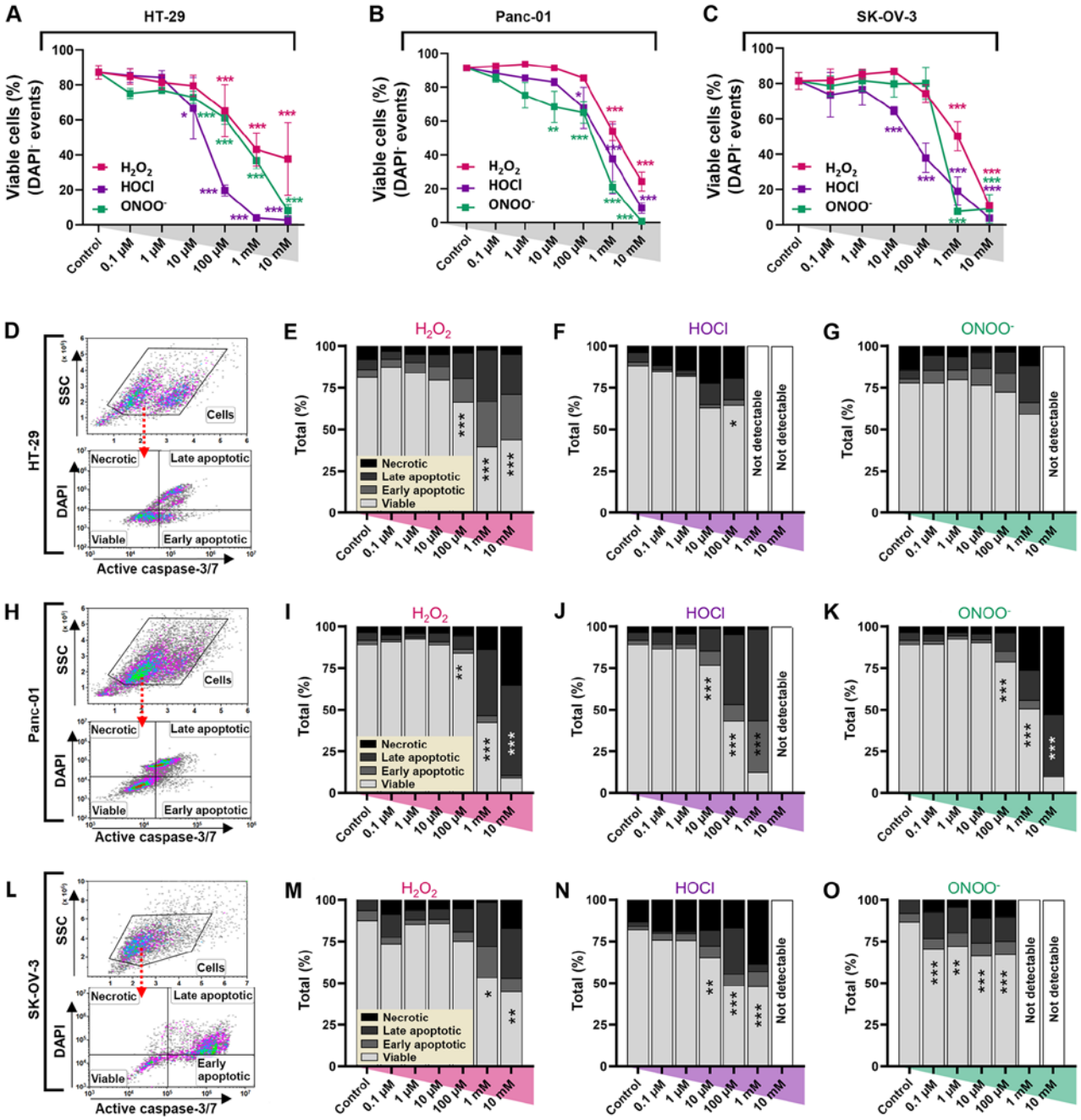

Figure 2. Oxidant treatment decreases viability of abdominal cancer cells *in vitro*. Percentage of viable (A) HT-29, (B) Panc-01 and (C) SK-OV-3 carcinoma cells at 24 h post-oxidant treatment. Data are presented as the mean ± SEM. (D) Representative forward scatter and SSC and DAPI and active caspase 3/7 dot plots of HT-29 cells analyzed via flow cytometry. Cytotoxic effects of (E) H_2O_2, (F) HOCl and (G) $ONOO^-$ in HT-29 cells. (H) Representative dot-plots of Panc-01 cells. Cytotoxic effects of (I) H_2O_2, (J) HOCl and (K) $ONOO^-$ in Panc-01 cells. (L) Representative dot-plots of SK-OV-3 cells. Cytotoxic effects of (M) H_2O_2, (N) HOCl and (O) $ONOO^-$ in SK-OV-3 cells. Data are from five (A-C) and 2-3 (D-O) independent experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. control. H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; $ONOO^-$, peroxynitrite; SSC, side scatter.

100 μM (Fig. 3B). In order to identify the role of antioxidant defense, a GSH tracer was used to assess the relative amounts of GSH in the intracellular compartment (Fig. 3C). A slight but non-significant decrease in cytosolic GSH levels was observed at concentrations ≤10 μM (Fig. 3D). Among the three types of ROS investigated, H_2O_2 exhibited the weakest effect on cytosolic GSH levels in HaCaT keratinocytes.

ROS exposure is concomitant with cytosolic and mitochondrial oxidation in cancer cells. Differences in oxidant capability to induce cell death were observed. High concentrations of HOCl and $ONOO^-$ exhibited the greatest toxicity and necrosis; H_2O_2 exhibited lower toxicity, and late apoptosis was observed at higher concentrations (Fig. 2). These differences indicated different underlying mechanisms for each ROS. In order to investigate this, intracellular oxidation was assessed. Cell pseudo-cytosolic DPC signal was used to segment the cell area via a software-based quantification tool (Fig. 4A) and the amount of bound and unbound GSH tracer, emitting at characteristic fluorescence emission spectra (Fig. 4B), inside the segmented cell region was quantified. Compared with untreated controls, ≥0.1 μM H_2O_2 and HOCl decreased intracellular GSH levels in HT-29 cancer

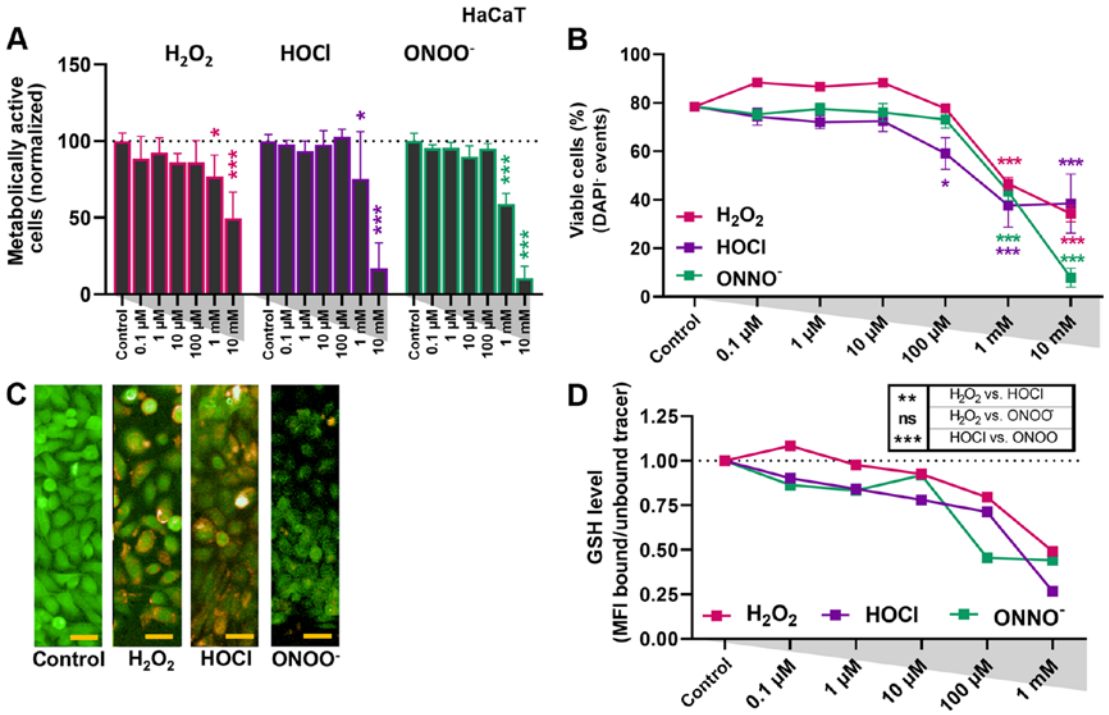

Figure 3. Oxidant treatment decreases the number of viable of HaCaT keratinocytes to a lesser extent than in abdominal cancer cells. (A) Percentage of metabolically active oxidant-treated HaCaT cells. Data are presented as the mean ± SD. (B) Percentage of viable HaCaT cells at 24 h post-administration of oxidants as analyzed by flow cytometry. Data are presented as the mean ± SEM. (C) Representative fluorescence microscopy images of both GSH fluorescence channels of HaCaT cells treated with 100 μM oxidants. (D) Quantification of GSH level in HaCaT cells at 24 h, normalized to untreated control. Data are from three (B) and 2-4 (A, C and D) independent experiments. Scale bar, 20 μm. *P<0.05, ***P<0.001 vs. control. H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; $ONOO^-$, peroxynitrite; GSH, glutathione; MFI, mean fluorescence intensity.

cells (Fig. 4C and D). $ONOO^-$ only notably decreased GSH levels at 1 mM (Fig. 4D). In Panc-01 cells, $ONOO^-$ and H_2O_2 decreased GSH levels at ≥0.1 μM, but to a lesser extent compared with HOCl at similar concentrations (Fig. 4F and G). Similar results were observed in SK-OV-3 cancer cells (Fig. 4I and J). Altogether, HOCl at low concentrations induced a notable consistent decrease in intracellular GSH levels in all three cancer cell lines, while non-malignant HaCaT keratinocytes were less affected at equimolar concentrations (Fig. 3D).

Following accumulation within the mitochondrial membrane, MTO fluorescence is increased upon oxidation via ROS (36). A notable increase in MTO fluorescence was observed with higher HOCl concentrations in all three cell lines (Fig. 4E, H and K). By contrast, both $ONOO^-$ and H_2O_2 failed to induce notable mitochondrial oxidation, although a small increase was seen with H_2O_2 at higher concentrations. Among the three types of ROS compared at equimolar concentrations, HOCl showed the most potent oxidative and cytotoxic effects in the cancer cell lines investigated.

HOCl and H_2O_2 increase inflammatory surface molecule expression levels in cancer cells. In addition to cytotoxicity, the inflammatory and immune-stimulating potential of anti-cancer agents is of interest in clinical and pre-clinical research. Translocation of certain damage-associated molecular patterns (DAMPs), such as CRT (the chaperon of the endoplasmic reticulum), serves as an 'eat-me' signal to promote the phagocytosis of dying tumor cells, leading to the promotion of antitumor immunity (34). For HT-29 cells, the three types of ROS at concentrations ≥1 μM increased expression levels of CRT on the membranes of viable cells (Fig. 5A and B). The most effective agent was HOCl, which induced a 30-fold increase in CRT at 10 mM (Fig. 5B). In dead HT-29 cells, H_2O_2 was most effective and significantly increased CRT expression levels at concentrations ≥1 mM (Fig. 5C). Changes in CRT expression levels in treated compared with untreated cells were smaller in dead compared with viable HT-29 cells. In viable and dead Panc-01 cells, HOCl did not significantly increase CRT expression levels. By contrast, both $ONOO^-$ and H_2O_2 at high concentrations promoted CRT levels, with H_2O_2 eliciting significantly elevated CRT levels, especially in dead cells (Fig. 5D-F). In viable SK-OV-3 cells, upregulation of CRT on the membrane was observed in a dose-dependent manner for all oxidative treatment regimens (Fig. 5G and H). However, this was only significant at 10 mM HOCl and $ONOO^-$. In the fraction of dead SK-OV-3 cells, significant CRT upregulation was observed at H_2O_2 concentrations ≥1 mM (Fig. 5I). Danger signals, such HSP70 and HSP90 (Fig. 5J and K), notably increased in HOCl-treated HT-29 and H_2O_2-treated Panc-01 cells. In dead SK-OV-3 cells, a notable increase in HSP90 was observed with all three types of ROS.

Discussion

In order to investigate ROS as a putative supplement to lavage treatment of PC, the present study assessed toxicity and immune-relevant surface marker expression levels in three human abdominal cancer cell lines (HT-29, Panc-01 and SK-OV-3) following exposure to H_2O_2, HOCl or $ONOO^-$. Cancer cells were more sensitive to ROS-induced toxicity compared with non-malignant HaCaT keratinocytes and exhibited increased levels of immuno-relevant surface markers. At

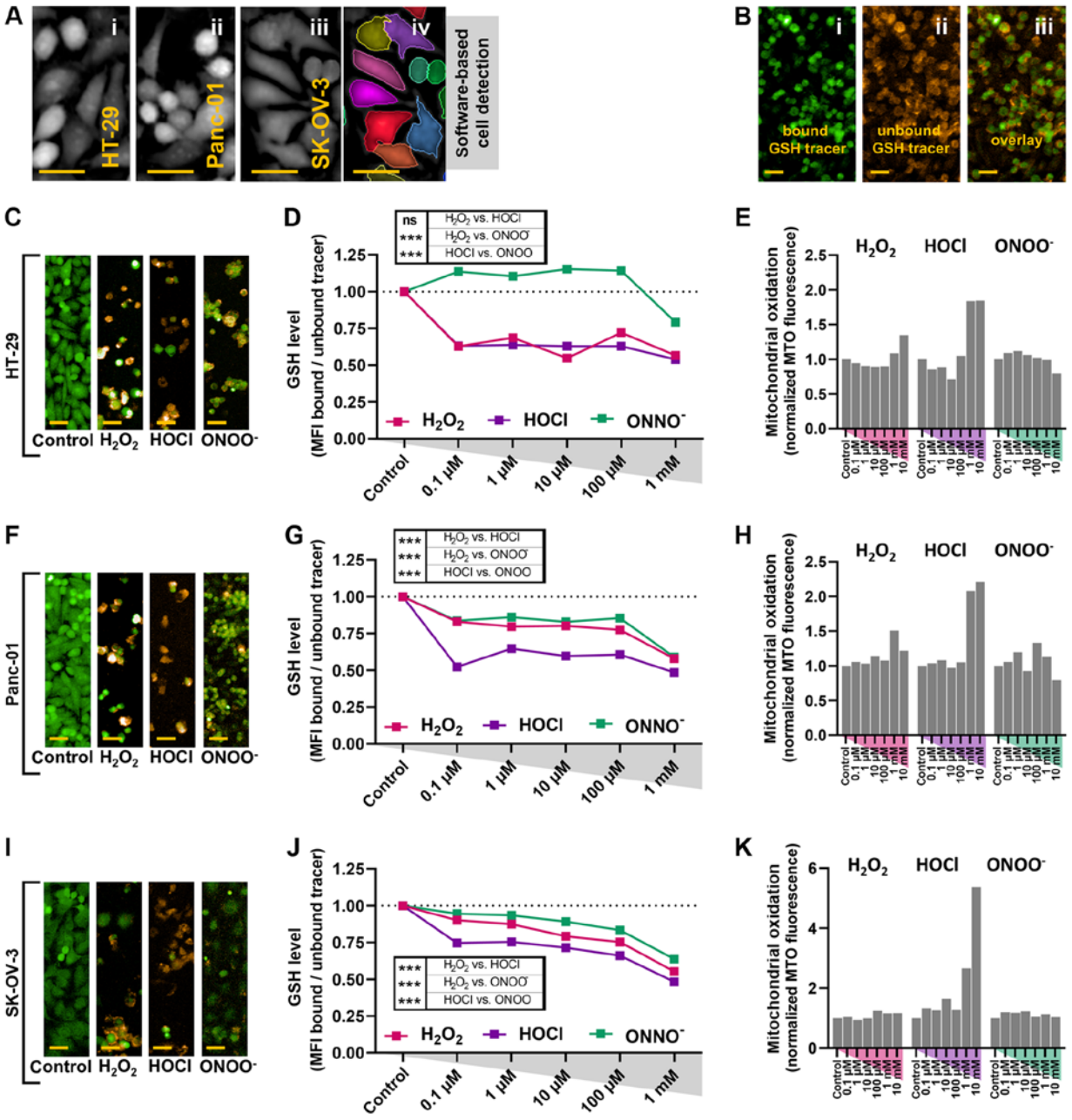

Figure 4. Oxidant treatment decreases intracellular GSH and increases mitochondrial ROS in cancer cells. (A) Representative images of the pseudo-cytosolic signal of i) HT-29, ii) Panc-01 and iii) SK-OV-3 carcinoma cells, with iv) representative software-based cell segmentation for subsequent quantitative analysis. (B) Representative images of cancer cells with i) bound and ii) unbound GSH tracer and iii) and overlay. (C) Representative images of GSH fluorescence channels for HT-29 cells in the presence or absence of 100 μM oxidants. (D) Quantification of GSH levels of HT-29 cells at 24 h. (E) Quantification of MTO fluorescence of HT-29 cells. (F) Representative images of GSH fluorescence channels for Panc-01 cells in the presence or absence of 100 μM oxidants. (G) Quantification of GSH levels of Panc-01 cells at 24 h. (H) Quantification of MTO fluorescence of Panc-01 cells. (I) Representative images of GSH fluorescence channels for SK-OV-3 cells in the presence or absence of 100 μM oxidants. (J) Quantification of GSH levels in SK-OV-3 cells at 24 h. (K) Quantification of MTO fluorescence of SK-OV-3 cells. Scale bar, 30 μm. Data are representative of two independent experiments with ≥50 fields of view per treatment condition, concentration and cell type. All data are normalized to untreated control. H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; $ONOO^-$, peroxynitrite; GSH, glutathione; ROS, reactive oxygen species; MTO, mitotracker orange; MFI, mean fluorescence intensity.

equimolar concentrations, HOCl appeared to be a particularly promising candidate for adjuvant ROS therapy.

The three types of ROS notably decreased the viability and metabolic activity in all three cancer cell lines, especially at concentrations ≥100 μM. Mechanistically, ROS may act via direct oxidation of intracellular proteins or nucleic acids, which promotes regulated cell death and senescence (37-39), as well as through ROS/reactive nitrogen species-redox signaling events that drive apoptosis via downstream signaling (40-42). At higher ROS concentrations, tumor cells were rendered inactivate but were not eliminated completely. This has previously been observed in H_2O_2-treated colorectal cancer cells (43), and similar effects have been detected in cells with functional ATM enzymes exposed to $ONOO^-$ (44), as well as HOCl in diabetes and liver disease (45). Here, HOCl and $ONOO^-$ were slightly more effective than H_2O_2 in decreasing the number of viable cancer cells, indicating different mechanisms of action for these molecules. It was previously shown

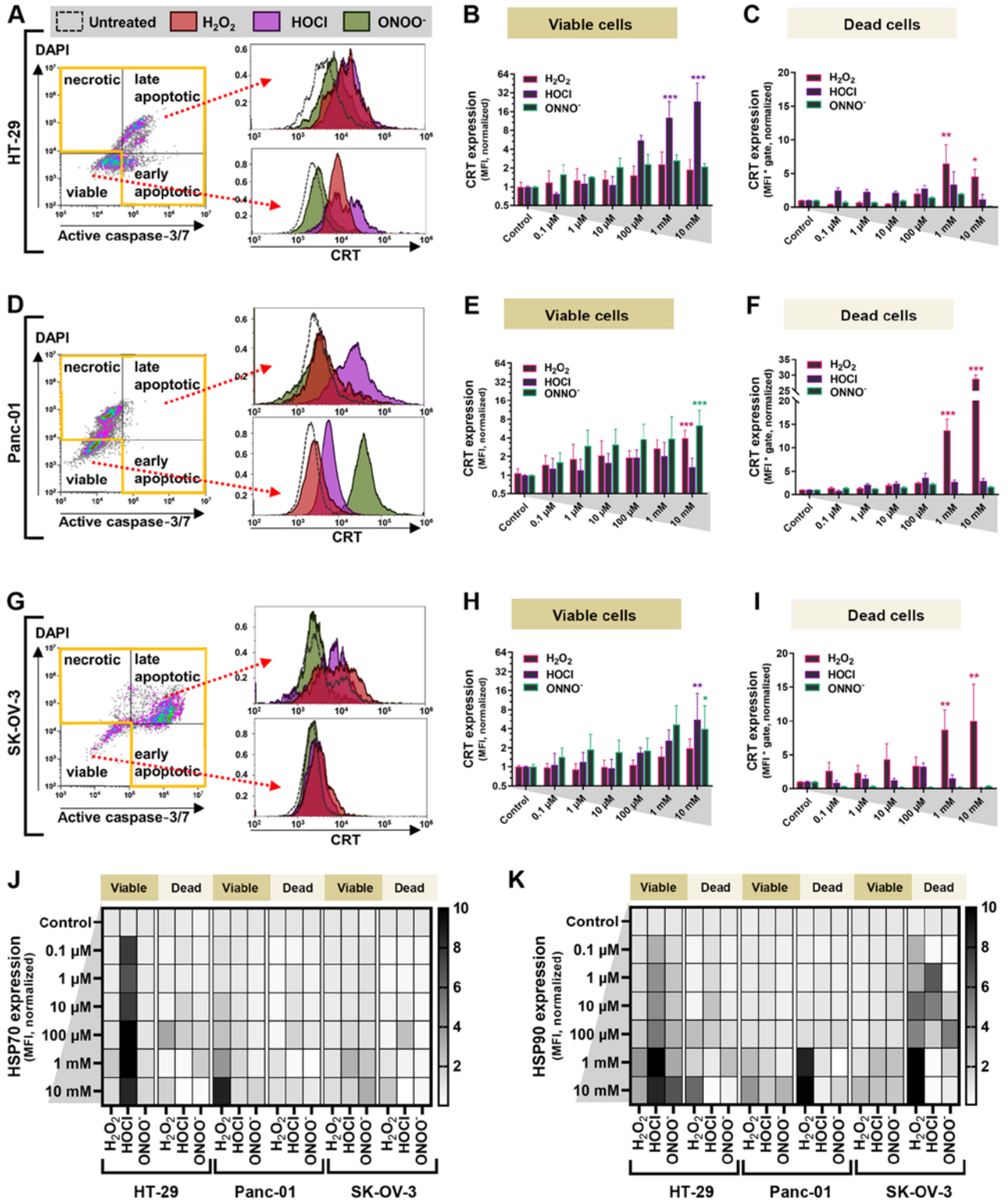

Figure 5. Oxidant treatment induces CRT expression in abdominal cancer cells. (A) Representative live-dead cell gating and overlay of CRT fluorescence in HT-29 cells treated with 100 μM H_2O_2 (red), HOCl (purple), $ONOO^-$ (green) or untreated (dotted line). (B) Fold-change in CRT expression levels in HT-29 cells. (C) Quantification of CRT expression levels in dead HT-29 cells. (D) Representative live-dead cell gating and overlay of CRT fluorescence in Panc-01 cells treated with 100 μM oxidants. (E) Fold-change in CRT expression levels in viable Panc-01 cells. (F) Quantification of CRT expression in dead Panc-01 cells. (G) Representative live-dead cell gating and overlay of CRT fluorescence in SK-OV-3 cells treated with 100 μM oxidants. (H) Fold-change of CRT expression levels in viable SK-OV-3 cells. (I) Quantification of CRT expression levels in dead SK-OV-3 cells. Heat map of expression levels of (J) HSP70 and (K) HSP90 in viable and dead cancer cells at 24 h. Data are from 3-4 independent experiments and are presented as the mean ± SD. All data are normalized to untreated controls. *P<0.05, **P<0.01, ***P<0.001 vs. control. H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; $ONOO^-$, peroxynitrite; CRT, calreticulin; HSP, heat-shock protein; MFI, mean fluorescence intensity.

that knockdown of the ROS-generating enzymes NADPH oxidase 1 (NOX1) and dual oxidase 1 in cancer cells prevents apoptosis following exposure to H_2O_2 (46). By contrast, knockdown of both NOX1 and inducible nitric oxide synthase

is required to abrogate HOCl-mediated apoptosis (47). These results suggest an underlying self-amplification mechanism of intracellular and membrane-generated ROS that contributes to ROS-induced cancer cell death. Moreover, $ONOO^-$ and H_2O_2 have been implicated in PI3K/Akt-mediated activation of the nuclear factor erythroid 2-related factor 2 pathway and microtubule-associated protein 1A/1B-light chain 3, indicating a role of antioxidant defense and autophagy in ROS-mediated cell death (48,49). HOCl is also produced endogenously by activated neutrophils via myeloperoxidase during inflammation to promote anti-microbial effects (50). In addition, HOCl promotes tumor cell death via several mechanisms, such as as the enhancement of antigen presentation and uptake, as well as the induction of an anti-tumor response driven by cytotoxic T cells (51).

The oxidation of cancer cells was here demonstrated by decreased intracellular levels of the antioxidant GSH. The availability of GSH provides information on the cellular antioxidant defense capacity and is associated with redox signaling processes (52). In the present study, non-malignant HaCaT keratinocytes showed the lowest overall decrease in GSH and the highest viability when exposed to different types of ROS at equimolar concentrations compared with the cancer cell lines tested. This may be because cancer cells naturally exhibit high levels of endogenous oxidative stress, which leads to increased susceptibility to ROS-induced cell death (14,53). The greatest decrease in intracellular GSH levels was observed following the administration of HOCl and H_2O_2, compared with $ONOO^-$. This may be explained by the lower affinity of the latter to GSH, the generation of S-nitroglutathione and the high affinity between $ONOO^-$ and CO_2 in reactions at high-rate constants (54,55). Here, $ONOO^-$ was also shown to have the lowest capacity to oxidize mitochondria. These findings are consistent with a previous study (46) that suggested HOCl to be of particular importance in the induction of cell death due to its ability of auto-propagating ROS formation through mitochondria.

One of the critical hallmarks of antitumor immune responses is the induction of ICD via increased danger signals (such as DAMPs), including the chaperon of the endoplasmic reticulum, CRT, on the tumor cell membrane (34,56). CRT can act as a danger and 'eat-me' signal when recognized by innate immune cells, such as dendritic cells (57). Other DAMPs include HSP70 and HSP90 as markers for cellular stress, leading to their upregulation and translocation to the cell membrane (58). ICD is associated with the upregulation of these molecules, as previously shown using antitumor agents tested for their immunogenicity in mice (34). In the present study, all types of ROS tested increased the levels of CRT on tumor cells. Specific responses, however, were dependent on the cell type and oxidant. HOCl, and to a lesser extent $ONOO^-$, gave promising results in at least two of the three tumor cell lines investigated by enhancing the presentation of pro-immunogenic molecules on their membrane. Exposure to these ROS results in the formation of DAMPs, which promote antitumor immunity (59,60), although the present study did not directly investigate the immunological consequences. Increased CRT levels were also observed in dead tumor cells following treatment with high concentrations of H_2O_2. H_2O_2 was previously found to promote a pro-immunogenic phenotype in murine colorectal cancer cells *in vitro*, as well as in mice with PC (37); this was concomitant with enhanced immune infiltration and activation. Moreover, ROS are capable of shaping activation profiles in human myeloid cells (37,61,62). ICD was previously observed with photodynamic therapy, which generates singlet oxygen to promote cellular oxidation (63,64), supporting the findings of mitochondrial oxidation and decreased cellular GSH levels in the present study. A previous report noted that $ONOO^-$ affects major histocompatibility complex-I recognition by T lymphocytes (65).

The present study identified HOCl as the most promising candidate in terms of toxicity and induction of immunogenic danger molecules (such as CRT) in cancer cells. HOCl was previously shown to enhance the immunogenicity of colorectal cancer cells *in vivo*, prevent distant metastasis of human melanoma cells, and alter antigen-presenting machinery and the cross-priming of tumor material (66-70). This makes HOCl a promising candidate as an adjuvant in peritoneal cancer therapy outside the current vaccination strategies against ovarian cancer employed with this type of ROS (71).

Treatment with H_2O_2, HOCl and $ONOO^-$ led to intracellular oxidation and notable toxicity in all abdominal cancer cell lines tested. Non-malignant HaCaT keratinocytes were less affected compared with cancer cells, suggesting a degree of specificity to the ROS-induced cell death. Treatment of cancer cells with these ROS also led to an upregulation of molecules associated with activation of immune cells. HOCl was the most promising therapeutic candidate, as it exhibited the greatest ability to inactivate cancer cells and upregulate danger molecules known to promote antitumor immunity. Future studies may extend this concept to provide novel therapeutic avenues in the treatment of PC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EF and SB designed the study. LM performed the experiments. EF, LM and SB confirm the authenticity of all the raw data. EF, LM, MBS and SB analyzed the data, prepared the figures and wrote and reviewed the manuscript. EF, LM, MBS and SB read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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