

2018

## Hydrogen Peroxide and Beyond - the Potential of High-Voltage Plasma Activated Liquids Against Cancerous Cells

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### Recommended Citation

Bourke, P. et al. (2018)Hydrogen peroxide and beyond - the potential of high-voltage plasma activated liquids against cancerous cells, *Anti-Cancer Agents in Medicinal Chemistry*, 2018, 18, 1-9 DOI: 10.2174/1871520617666170801110517

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Funder: Science Foundation Ireland

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# Hydrogen peroxide and beyond - the potential of high-voltage plasma-activated liquids against cancerous cells

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**Abstract:** The use of plasma-activated liquids such as PBS, medium or simply plasma-activated water (PAW) has been receiving increasing attention for applications in cancer treatments. Amongst the reactive species contained in these solutions, hydrogen peroxide appears to play a pivotal role in causing cytotoxic effects. While H<sub>2</sub>O<sub>2</sub> concentrations can be correlated with reduced cell viability and growth and used as an indicator of the potential efficacy of a plasma-activated water, comparisons to standard H<sub>2</sub>O<sub>2</sub> kill curves demonstrate a potency in PAW which exceeds H<sub>2</sub>O<sub>2</sub> associated toxicity, indicating that other plasma-generated species play an important role.

Using a high-voltage dielectric barrier atmospheric cold plasma (DBD-ACP) system, we demonstrate the generation of plasma-activated water with high cytotoxic potential and good storage stability. The potency of the activated solutions can be modulated using system or process characteristics such as voltage level, treatment time and post-treatment storage time and target-related characteristics such as surface to volume ratio. All of these parameters were found to impact cell viability in a hydrogen peroxide concentration correlated manner. The susceptibility of two cancer cell lines to PAW was similar to that observed for two non-cancer cell lines and the toxicity of plasma-activated water exceeded that of the corresponding hydrogen peroxide concentrations.

This study examines the role of H<sub>2</sub>O<sub>2</sub> in PAW-mediated cytotoxic effects on different mammalian cells lines and investigates effects beyond H<sub>2</sub>O<sub>2</sub> employing a set-up where short-lived reactive species can be discounted and activated liquids with long-term stability are generated. Here we investigate the cytotoxic mediators generated in water specific to high-voltage DBD-ACP.

**Keywords:** plasma activated water, ROS, RNS, cytotoxicity, hydrogen peroxide, peroxynitrite, peroxynitrate.

## 1. INTRODUCTION

Solutions exposed to plasma discharges provide a novel resource which can be tailored to a range of applications in medicine and healthcare. Cytostatic or cytotoxic effects of plasma activated liquids in mammalian systems have sparked interest in their investigation as novel cancer therapeutics and efficacy has been demonstrated in a number of different cancer cells including glioblastoma, ovarian, bladder cancer and lung cancer [1-5]. However, a holistic insight into the mechanisms governing these liquid-mediated effects of plasma exposure and the main chemical effector species is required to release the full potential of this technology, to develop possible applications and to inform safety considerations depending on the cell type and application in question.

Investigations of non-thermal plasma in cancer treatment to date have largely been performed by exposing cells directly to plasma discharge or plasma afterglow, where a range of plasma devices have been utilized on various cancerous cells [6, 7]. The ionization of gas in a plasma discharge produces a range of reactive oxygen and reactive nitrogen species (ROS/RNS) such as ozone, atomic oxygen, superoxide

anion, hydroxyl radical, hydrogen peroxide and nitric oxides [8]. These reactive species can react with proteins, lipids and nucleic acids and cause damage to the cellular envelope and intracellular components including enzymes and DNA.

Increased intracellular levels of ROS and RNS can lead to mitochondrial dysfunction, cell cycle arrest, altered cell signaling and apoptosis [6, 7] and both extra and intracellular ROS scavengers have been shown to protect cells from cytotoxic/anti-proliferative effects [9]. In situ generation of OH radicals through the Fenton reaction may be one of the main causes of plasma-induced cell death derived from O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> in plasma effluents and plasma treated liquids [10, 11]. Yet ROS-independent mechanisms of cell death in response to non-thermal plasma have also been described [12].

Many of the effects of plasma treatment (the major exceptions being electric field and UV radiation) are mediated through reactive species in liquid at the cell – plasma interface, thus many of the effective observed phenomena and pathways are likely to be implicated in the action of plasma activated liquids.

The exposure of aqueous solutions to atmospheric plasmas results in the generation of secondary products such as hydrogen peroxide, nitrates and nitrites which may react to form further cell toxic compounds such as peroxynitrite/

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peroxynitrous acid. A dominant function for H<sub>2</sub>O<sub>2</sub> in the cytotoxic effects of plasma-activated liquids has been established through a number of studies [1, 13-18] but, importantly it has also been shown that this compound is not the sole toxic mediator. Hydrogen peroxide can induce DNA damage, cause cell cycle arrest and trigger apoptosis and its toxicity is determined largely by the cellular anti-oxidant status and ability to detoxify H<sub>2</sub>O<sub>2</sub> through catalase.

The cytotoxic effects of plasma activated medium (PAM) have been suggested to be selective for cancer cells in some studies including glioblastoma cells and ovarian cancer cells, and exposure to PAM inhibited the growth of tumour spheroids in vitro while PAM injection into subcutaneous tumours in mice resulted in a reduction of tumour size in vivo [18-20].

This purported selectivity for cancerous cells has been proposed to result from increased baseline endogenous ROS levels in these cells resulting from aberrant metabolism [9] or an increased surface expression of aquaporins which results in greater uptake of reactive species such as H<sub>2</sub>O<sub>2</sub> [5, 21] but definite selectivity remains to be shown [22]. More recently, it has been argued that the main potential of plasma in cancer therapy may lie in propagation of the effects through bystander effects on the surrounding tissue and the activation of the immune system through immunogenic cell death (ICD) [23].

Much work has been performed on the identification of reactive species in plasma activated liquids and their resulting cytotoxic effects. It is increasingly evident that very different chemistries can prevail depending on the type of plasma system used, the gas in which discharge occurs as well as a range of device-based parameters and target characteristics [24]. Water becomes acidified during plasma treatment and H<sub>2</sub>O<sub>2</sub>, nitrites and nitrates are reactive species most commonly detected. However, their concentrations can vary strongly from undetectable to mM ranges depending on device configuration or working gas. In plasma activated water generated using indirect DBD discharge, Traylor and colleagues reported detection of H<sub>2</sub>O<sub>2</sub> around 100µM and nitrites and nitrates of 1-5mM [25]. The composition of PAW generated with a gliding arc depended on the configuration of the gliding arc reactor used and the working gas, where H<sub>2</sub>O<sub>2</sub>, nitrites and nitrates were obtained in varying concentrations from undetectable to mM [26].

Using an RF plasma-jet, Wende and co-workers showed H<sub>2</sub>O<sub>2</sub> dominated cytotoxicity when using argon and humidified argon as feed gas but suggest atomic oxygen to mediate toxicity in argon/air and argon/oxygen mixtures where H<sub>2</sub>O<sub>2</sub> is only found in trace amounts [27]. The choice of target liquid furthermore modulates reactive species production through buffering capacities (e.g. PBS), scavenging potential (antioxidants in cell culture medium) or selective formation of secondary reactive species. Atomic oxygen for example has been proposed to react with chloride ions in saline to form cytotoxic ClO<sup>-</sup> or Cl<sub>2</sub><sup>-</sup> [27]. In a recent study, Girard and colleagues demonstrated the synergistic effect of H<sub>2</sub>O<sub>2</sub> and nitrite in killing normal as well as cancerous cells and conclude a similar mechanism of action for the effects of a He plasma jet [16]. The cytotoxic effects of plasma-activated medium depend on the amino acids present, on scavengers such as pyruvate and can be modulated through the addition of FBS [1, 28-30].

Therefore, a generalization on the identity of cell-toxic effectors in plasma-activated liquids becomes difficult. In this study we focus on the properties and cytotoxic mediators of plasma activated water generated using a high-voltage dielectric barrier discharge system where plasma discharge is generated in air. A contained post-treatment storage time (PTST) significantly increases the efficacy of the PAW and suggests the longer-lasting species as key effectors.

## 2. MATERIALS AND METHODS

### 2.1. Plasma system

Plasma treatment was performed using a high-voltage dielectric barrier discharge atmospheric cold plasma system custom built at Dublin Institute of Technology (Dublin, Ireland) with a maximum output of 120 kV<sub>RMS</sub> at 50 Hz which has been described in detail [31, 32].

### 2.2. Plasma treatment

Deionized water was placed in the discharge area in multi-well plates or petri-dishes inside a polypropylene container and sealed with air-tight film. The samples were placed either directly in the area of discharge between high voltage and ground electrode (in-field, IF) or within the sealed box but outside of the area of discharge (out-field, OF). Plasma was generated at 80 kV<sub>RMS</sub> in air, solutions were subjected to 24 hour post treatment storage time at room temperature before opening unless otherwise specified. Plasma activated water was subsequently stored at 4°C.

### 2.3. Chemical analyses

Unless specified otherwise all reagents were purchased from Sigma-Aldrich, Arklow, Ireland. Hydrogen peroxide concentrations in solution were determined using oxidation of potassium iodide to iodine and spectrophotometric measurement at 390 nm or reaction with sulfuric acid solution of TiOSO<sub>4</sub>, measured at 405nm. A standard curve of known hydrogen peroxide concentrations was included on each plate and used to convert absorbances into peroxide concentrations. pH measurements were performed using a Orion pH meter model 420A (Thermo Fisher Scientific).

Nitrite concentrations were determined using Griess reagent and spectrophotometric measurement at 548nm after 30min incubation and compared to a sodium nitrite standard curve. Nitrate concentrations were assessed using a nitrate spectrophotometric assay kit (Merck Chemicals, Darmstadt, Germany).

UV absorption spectra were obtained with a Shimadzu UV-1800 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan).

### 2.4. Cell culture

Cell lines were obtained from culture stocks of Dublin Institute of Technology or University College Dublin. The following established human cancerous cell lines were used: human cervical carcinoma HeLa and human glioblastoma U373MG; and the human immortalized human keratinocyte HaCaT. Chinese hamster ovary (CHO-K1) cells were included as a further non cancer line for which the DBD plasma device has previously been characterized with regard to cytotoxic and genotoxic effects. Cells were cultured in DMEM/F12 medium supplemented with 2 mM L-glutamine and 10% foetal bovine serum (FBS). Cells were grown at

37°C and 5% CO<sub>2</sub> in a humidified incubator. Cell concentrations and viability were assessed using trypan blue counting.

## 2.5. Cytotoxicity assays

Cells were seeded at 2.5x10<sup>4</sup> cells/ml in DMEM/F12 + 10% FBS supplemented with 20% of plasma activated water or respective H<sub>2</sub>O<sub>2</sub> dilutions. Cell growth was assessed through quantification of cell mass by staining of adherent cells with crystal violet after 3-4 days in culture. Culture supernatant was removed and cells were fixed with 70% methanol for 1 min followed by staining with 0.2% crystal violet solution for 10 min. After extensive washes with water, plates were air-dried and the crystal violet was solubilized with 10% acetic acid and absorbance measured at 560 nm on a spectrophotometric microplate reader (Biotek, Winooski, USA). Cell growth was expressed as percentage of control cells grown in medium supplemented with untreated water.

## 2.6. Statistical analysis

Results represent the means of triplicate determinations. Standard deviations and statistical analysis where applicable were performed by analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software Inc., La Jolla, USA).

# 3. RESULTS AND DISCUSSIONS

## 3.1. Plasma activated water

Plasma activated water was generated inside a sealed container either through direct plasma exposure by placing water samples in-field of the area of discharge or indirect treatment where samples were exposed to plasma-reactive species in the treated air but were outside of the actual area of discharging (out-field).

Plasma-activated water generated using a high-voltage DBD system contained high concentrations of hydrogen peroxide but no detectable amounts of nitrite. The pH decreased to 2-3 and remained stable. The concentrations of H<sub>2</sub>O<sub>2</sub> produced could be modulated through the applied voltage, treatment time and also post-treatment storage time (PTST) specific to the contained plasma treatment set-up employed here (Fig. 1) and remained stable over storage for several weeks at 4°C [13]. The stability of H<sub>2</sub>O<sub>2</sub> concentrations in plasma-activated solutions over time has been shown to depend on the absence of scavenging factors such as pyruvate or certain amino acids such methionine and cysteine [1, 33]. Introduction of post-treatment storage periods allows longer contact times for gas reactive species to react with and diffuse into the liquid phase. As demonstrated previously for this system high gas phase levels of ozone disappear within the 24h PTST [34].

H<sub>2</sub>O<sub>2</sub> concentrations were dependent on the surface area to volume ratio of the treated sample and increased with higher surface areas available for interaction with the gas phase for both in-field and out-field (Fig. 2). Similarly, higher concentrations of H<sub>2</sub>O<sub>2</sub> were detected with increasing well diameter for plasma-activated medium, which translated to stronger cytotoxic effects in studies by Yan et al [30].

## 3.2. Cytotoxic effects

Hydrogen peroxide has known cytotoxicity on mammalian cells but IC<sub>50</sub> values diverge greatly between different cell lines, depending on cellular redox status and the cell's ability to detoxify H<sub>2</sub>O<sub>2</sub> through catalase. For the cell lines tested here, a greater than 3-fold difference was found between the most susceptible (HaCaT) and the most resistant (U373mg) (Fig. 3 and Table I).

Cell growth in response to supplementation with plasma activated water (20% v/v) correlated with the solutions hydrogen peroxide concentrations in a dose dependent-manner showing a characteristic response-inhibition curve (Fig. 4). However as reported previously the corresponding IC<sub>50</sub>s were in most cases strikingly lower than those obtained through H<sub>2</sub>O<sub>2</sub> supplementation of the medium (Table 1). Moreover, the highly H<sub>2</sub>O<sub>2</sub>-resistant glioblastoma cell line U373mg (IC<sub>50</sub> > 300 μM) showed an IC<sub>50</sub> in PAW which was comparable to the other cell lines. In agreement with numerous studies, these observations confirm that mammalian cytotoxicity correlates with hydrogen peroxide content in plasma activated solutions but that this does not, however, constitute the only cytotoxic factor [13, 16].

## 3.3. The role of pH

Non-buffered solutions become acidified during plasma treatment and this reduced pH has been shown to be crucial for cell toxic effects of plasma activated solutions on bacterial cells [35]. The addition of PAW to DMEM/F12 medium, which is buffered both with HEPES and bicarbonate and contains 10% FBS, does not result in an acidification of the overall solution. The medium is able to buffer the addition of PAW up to concentrations of a total of 80% (v/v) and significant changes in pH of the medium do not occur until an addition of HCl up to a total concentration of 10mM (Fig. 5a,b). In comparison, PAW is added only up to a total volume of 20% (v/v) or an equivalent of 0.1-1mM of H<sup>+</sup> for a pH of 2-3. Any pH related effects of PAW when diluted in DMEM can hence be discounted.

To exclude the possibility that acidic species in PAW are required for reactions with medium components to form other toxic -albeit pH neutral- reaction products- PAW was pH neutralized through the addition of concentrated PBS before addition to cell culture medium. The effects on cell growth did not differ between cultures supplemented with pH buffered PAW or PAW diluted respectively with H<sub>2</sub>O and comparable cytotoxic effects were elicited by both (Fig. 6).

## 3.4. The role of nitrites and nitrates

Nitrites were not generated in detectable concentrations in contrast to many plasma activated liquids generated using jet-based systems. Supplementation of cell cultures with nitrite at concentrations up to 1200μM did not show cytotoxic effects on the cell lines tested and nitrite *per se* can thus be excluded as playing a role in the inhibition of cell growth (Fig. 7) in agreement with observations made by others [27]. Nitrate was generated in plasma-activated water in a treatment time dependent manner, reaching up to 600μM. However, no cytotoxic effects of nitrate were found in this range or for 10-fold higher concentrations in the mammalian cells tested (Fig. 7b).

Hydrogen peroxide reacts with nitrite to reaction products such as nitrate ( $\text{NO}_3^-$ ) and peroxyxynitrite ( $\text{ONOO}^-$ ). As shown in Fig. 8, titration of  $\text{H}_2\text{O}_2$  with sodium nitrite results in a loss of  $\text{H}_2\text{O}_2$  signal as detected using  $\text{TiOSO}_4$  (Fig. 8b), but insubstantial changes only in the oxidative signal detected using KI (Fig. 8a). Peroxyxynitrous acid can be generated through reaction of  $\text{H}_2\text{O}_2$  and nitrite at acidic pH and may be the oxidative species which reacts with the potassium iodide. Sodium nitrate of concentrations from  $60\mu\text{M}$  to  $6000\mu\text{M}$  showed no reaction with potassium iodide. Peroxyxynitrite/peroxyxynitrous acid is a secondary reactive species produced in plasma exposed liquids and - as a known cell toxic agent - has been discussed to be responsible for biocidal effects in both microbial and mammalian cell systems [25, 36-38]. The generation of peroxyxynitrite has been indicated in various experimental set-ups but its detection is complicated by its inherent instability and a lack of specific assays due to cross-reactivity of other ROS/RNS. The transient formation of peroxyxynitrite has been demonstrated in PBS at alkaline pH treated with  $\text{He}/1\%\text{N}_2$  plasma [39]. At neutral pH and  $37^\circ\text{C}$  this species has a half-life of 1 sec [40] and would decompose instantaneously upon dilution in cell culture medium. On the other hand, with direct plasma treatment of cells or tissues, the generation of peroxyxynitrite and subsequent reactions with bio-molecules may play an important role in plasma-induced cell damage. Reactions with components of the cell culture medium are possible and could result in oxidation or nitration of protein residues.

Cells supplemented with nitrite at concentrations from  $6\mu\text{M}$  to  $100\mu\text{M}$  and  $65\mu\text{M}$   $\text{H}_2\text{O}_2$  responded to the toxic effects of hydrogen peroxide but indicated no increased toxicity of nitrite and  $\text{H}_2\text{O}_2$  combined (Fig. 9). These findings are in agreement with observations by Wende and co-workers who tested  $125\mu\text{M}$  nitrite against a range of  $\text{H}_2\text{O}_2$  concentrations and found no impact on the  $\text{IC}_{50}$  [27]. A synergy of  $\text{H}_2\text{O}_2$  and nitrite in cytotoxic effects on both normal and malignant cells was, however, reported by Girard and colleagues who suggested this synergy as a basis for the cytotoxicity of plasma treatment with a Helium jet [16].

UV spectra of plasma activated water displayed an absorption peak between 200 and 220nm which increased with treatment time. While this lies in the absorption region of both nitrite and nitrate (Fig. 10, 11), no nitrite was detectable by chemical analysis. Nitrate was detected in concentrations ranging up to  $600\mu\text{M}$  and can be assumed as a main contributor to the observed spectrum.

The formation of peroxyxynitric acid as a decomposition product of peroxyxynitrite at neutral pH has been stipulated [41]. Kitano and co-workers, who successfully isolated and characterized peroxyxynitric acid using low temperature, low pH ion exchange chromatography [42], recently proposed it as the major biocidal reactive species in plasma treated water at the International Conference for Plasma Medicine 6 (ICPM6). Peroxyxynitric acid/peroxyxynitrate (PNA) and peroxyxynitrous acid/peroxyxynitrite (PNI) possess similar 2 electron oxidative behavior but differ in their stability as a function of pH and  $\text{CO}_2$  [43]. While the formation of peroxyxynitrate from decomposition of peroxyxynitrite at neutral pH to alkaline pH has been shown, it can also be formed through the reaction of  $\text{O}_2^-$  with  $\text{NO}_2$  [44], and these are reactive species which can be generated in plasma discharge

in air. The analysis of the gas phase during discharge of the DBD system used here has shown the generation of  $\text{NO}_2$  while high concentrations of ozone were measured after treatment but decreased to undetectable levels within 24 hours [31, 34]. The superoxide anion radical is one of the products in the chain reaction of ozone decomposition in water and could react with the  $\text{NO}_2$  in a sealed environment. Generation of peroxyxynitric acid in solution requires extreme reaction conditions such as  $> 90\%$   $\text{H}_2\text{O}_2$  and  $70\%$   $\text{HNO}_3$  or pulse radiolysis of oxygen saturated  $\text{NaNO}_2$  or  $\text{NaNO}_3$  making it a difficult species to study [45]. Gupta and co-workers have described the formation of peroxyxynitric acid with an absorption maximum at 284nm [41].

Examination of UV spectra obtained of plasma activated water used here, indicates an absorption peak around 280nm in some samples (Fig. 11b), a peak observed for neither  $\text{NaNO}_2$  nor  $\text{NaNO}_3$  (Fig. 10b). Whilst further detailed chemical analysis is required, the possible generation of peroxyxynitric acid/peroxyxynitrate through high voltage treatment of water is proposed as a source of reactive species with cytotoxic activity in addition to  $\text{H}_2\text{O}_2$  in the plasma activated water used here.

## CONCLUSION

Despite the intensification of research on the effects of plasma-activated liquids on mammalian cells and cancerous cells in particular towards potential applications in cancer therapy, the exact cause-effect relationships of 'plasma-liquid-cell' remain incompletely elucidated.

A myriad of reactive species and biologically relevant factors are generated in plasma discharges. These differ in response with the plasma device employed and its operating parameters. It is therefore not surprising that a range of different reactive species have been identified in plasma-treated liquids where the choice of liquid adds a further degree of variability. Importantly, it may be within this diversity that the distinct potential of plasma-activated liquids for inducing cell death of cancerous cells lies. Eukaryotic cells possess an arsenal of mechanisms to deal with potentially fatal stress resulting from ROS and RNS. These include anti-oxidants such as glutathione, thioredoxin and highly specialized enzymes such as catalase and superoxide dismutase. Providing challenges on multiple fronts may overcome these cellular defenses and result in an accumulation of damage that ultimately halts cell cycle progression and leads to apoptotic cell death. Providing a challenge that activates multiple cell defences may also be less prone to the development of resistances.

In cytotoxic plasma activated water an essential role for  $\text{H}_2\text{O}_2$  has been demonstrated multi-fold, yet further contributing factors are apparent and remain to be identified. In this study, the post-treatment storage time at room temperature for 24 hours not only enhanced the cytotoxic potency of the PAW but this also rules out short-lived factors as causative agents. We hypothesize that a cytotoxic agent produced using the DBD set-up described here, which acts additively/synergistically with  $\text{H}_2\text{O}_2$ , could be peroxyxynitric acid/peroxyxynitrate.

It remains to be established with certainty that PAW selectively inhibits cancerous cells by comparison with normal cells. However, an unspecific cytotoxic effect on dividing cells may

well suffice to make this product relevant to further cancer-therapeutic research, where certain tumour cells could be targeted through localized application and retention. Using a well characterized high-voltage system with species containment we generate a cell-toxic liquid using the simplest of media available, i.e. water. The contained approach, previously applied for decontamination of packaged foods or medical products [46, 47], holds further promise not only for its ability to increase levels of stable cytotoxic effectors but could also generate a sterile product protected against contamination prior to its potential application.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

Financial contributions to this study were obtained through funding from Science Foundation Ireland Grant No 14/IA/2626. The authors are grateful to Prof. Mohamed Al-Rubeai (UCD) for provision of some cell lines used in this study.

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**FIGURE LEGENDS**

Fig. 1: Generation of hydrogen peroxide in water exposed to DBD discharge at 80kV, in-field, dependent on treatment time and post-treatment storage time (PTST). Post-treatment storage time was assessed for samples treated at 80kV for 10 min (dashed line).

Fig. 2: Hydrogen peroxide concentrations in plasma activated water depending on the volume to surface area ratios.

Fig. 3: Dose-response curves of cell lines U373mg, HeLa, HaCaT and CHO-K1 to hydrogen peroxide.

Fig. 4: Dose-response curves of cell lines U373mg, HeLa, HaCaT and CHO-K1 to supplementation with plasma activated water at 20% (v/v).  $H_2O_2$  in plasma activated water was determined using  $TiOSO_4$  and KI method and cell growth is shown as function of final concentration of  $H_2O_2$  after dilution in DMEM/F12 medium relative to cultures supplemented with pure water.

Fig. 5a,b: Buffering capacity of DMEM/F12 medium for addition of acidic plasma activated water (a) and 10mM HCl (b). Circle and square indicate the corresponding concentration range of plasma activated water supplementation used in cell culture experiments.

Fig. 6: Cell growth of HaCaT cells in response to supplementation with plasma activated water, pH-neutralized by addition of 4.5x PBS or control-diluted with water. Cells were supplemented with a total of 20% (v/v) plasma activated water and cell growth is shown as function of the respective final hydrogen peroxide concentrations.

Fig. 7a, b: Cell growth in response to supplementation with  $NaNO_2$  (a) and  $NaNO_3$  (b). Sodium Nitrite or nitrate solutions of 60-6000 $\mu$ M were added into the cell culture medium at 20% (v/v) resulting in final concentrations between 12 and 1200  $\mu$ M.

Fig. 8a,b: Reaction of sodium nitrite with hydrogen peroxide shows no change in oxidation of potassium iodide (a) but loses its capability to react with  $TiOSO_4$  when concentrations of nitrite are in excess (b). A matrix of  $NaNO_2$  and  $H_2O_2$  concentrations was set up ranging from 0-6000 $\mu$ M of each compound and each concentration of  $NaNO_2$  was mixed 1:1 with each concentration of  $H_2O_2$ .

Fig. 9: Cell growth in response to supplementation with  $NaNO_2/H_2O_2$  mixtures. Cells were supplemented at 20% (v/v) with  $NaNO_2$  (closed symbols) or  $NaNO_2$  and  $H_2O_2$  (open symbols).  $NaNO_2$  was used at a final concentration of 6-100 $\mu$ M,  $H_2O_2$  at 65 $\mu$ M. Cell growth is shown relative to control cultures with 20% v/v water.

Fig. 10: UV spectra of sodium nitrite (600 $\mu$ M, 300 $\mu$ M), sodium nitrate (600 $\mu$ M, 300 $\mu$ M), hydrogen peroxide (600 $\mu$ M) and mixtures thereof. Spectra for 300 $\mu$ M  $NaNO_2$  (dotted line) and a mixture of 600 $\mu$ M  $H_2O_2$  and 600 $\mu$ M  $NaNO_2$  (long dash) overlapped almost identically. Overview spectra shown from 190-400nm (a) and detailed spectra from 250-400nm (b).

Fig. 11: UV spectra of plasma activated water generated at different treatment and post-treatment storage times with in-field (IF) or out-field (OF) treatment. Overview spectra shown from 190-400nm (a) and detailed spectra from 250-400nm (b).

FIGURES

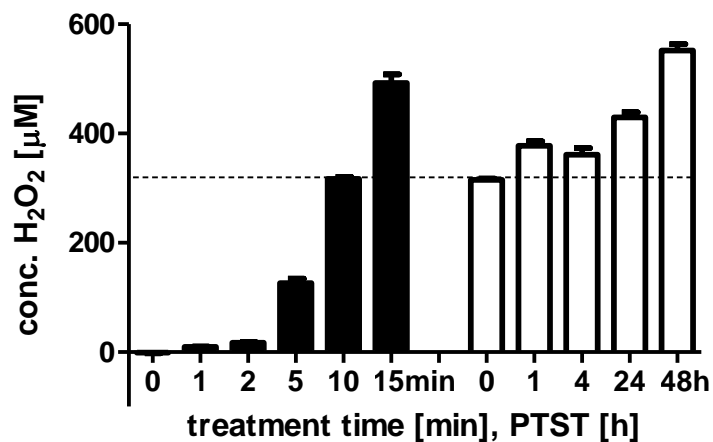


Figure 1

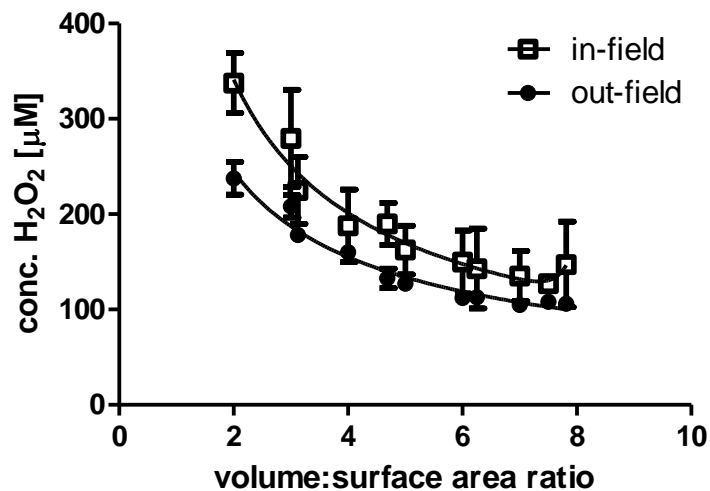


Figure 2

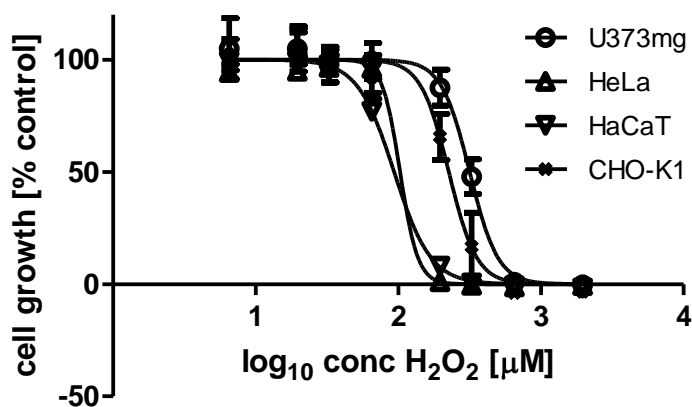


Figure 3

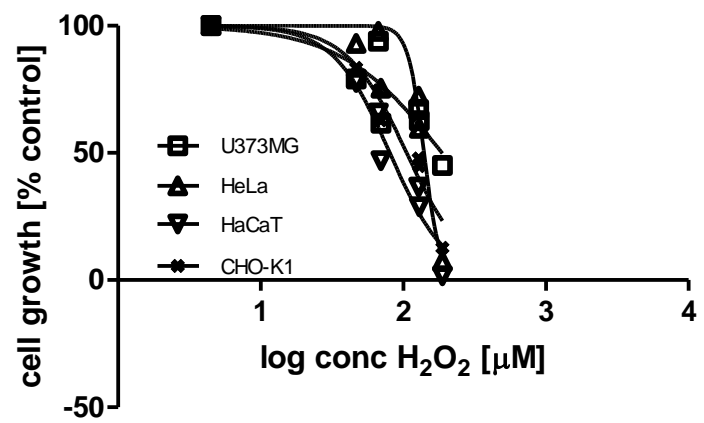
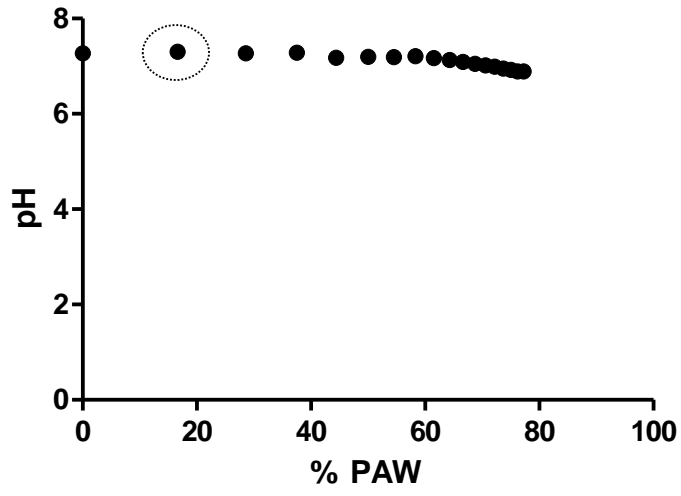
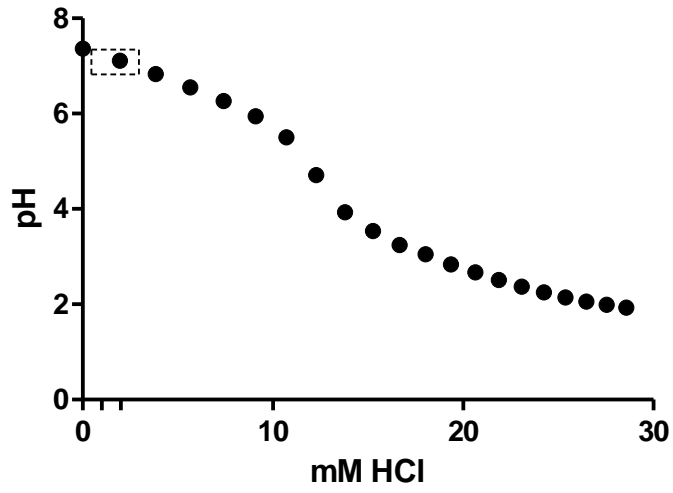


Figure 4



a



b

Figure 5a,b

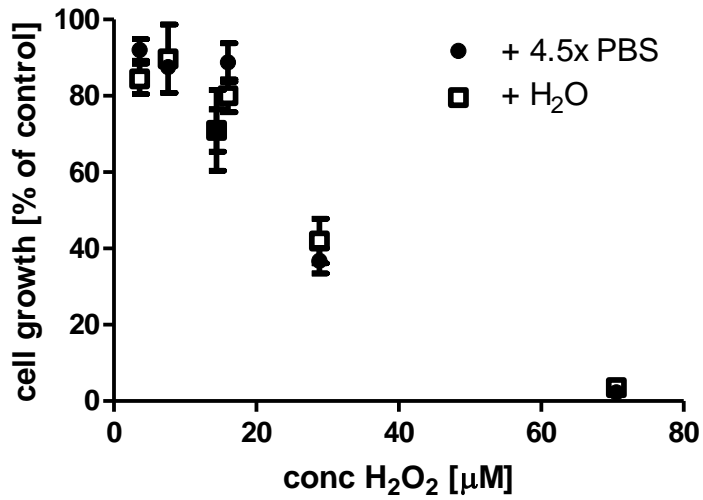
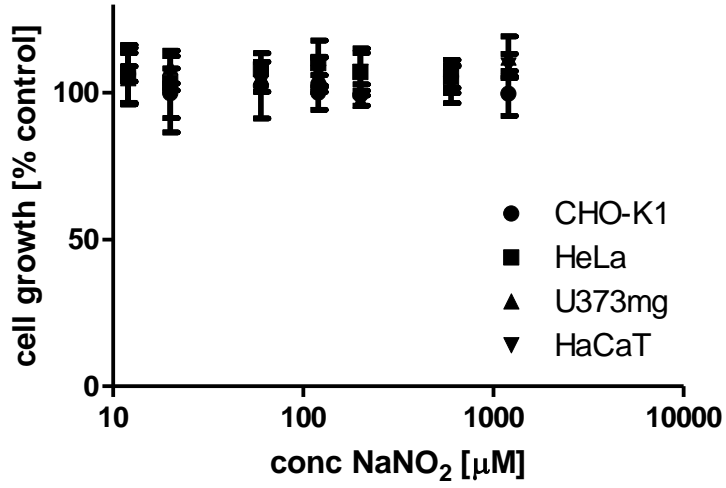
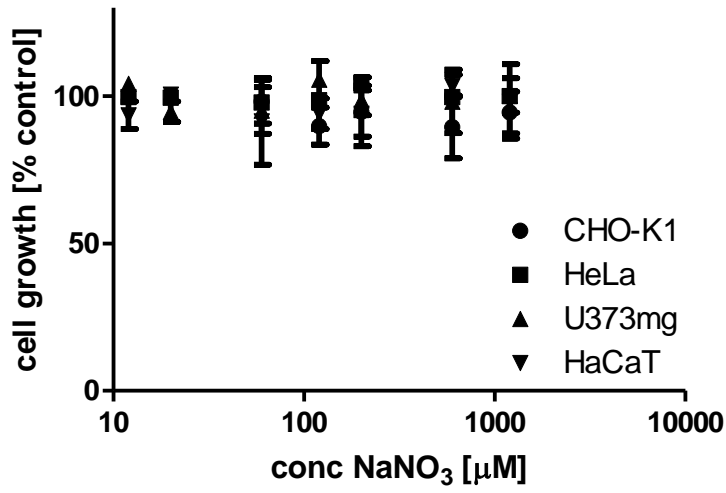


Figure 6



a



b

Figure 7a,b

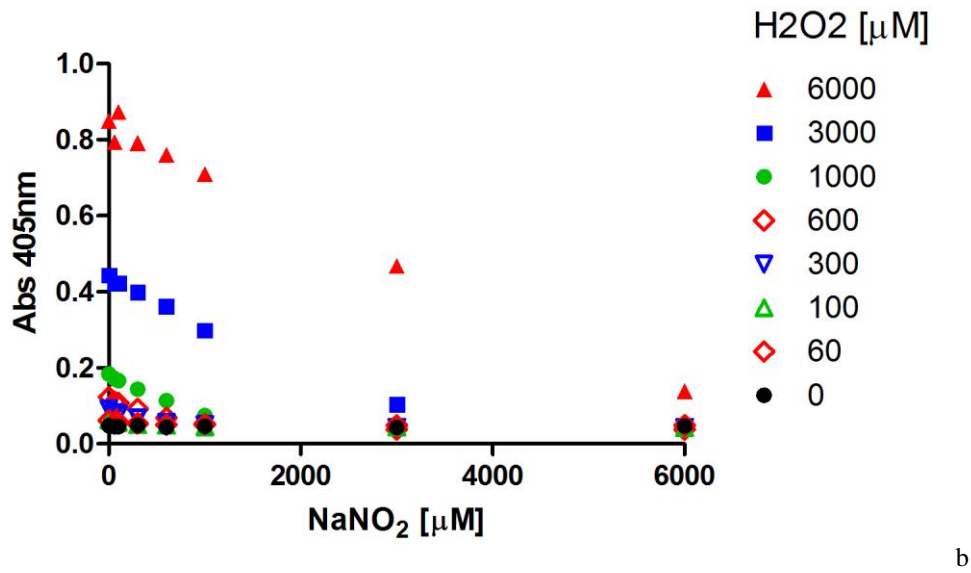
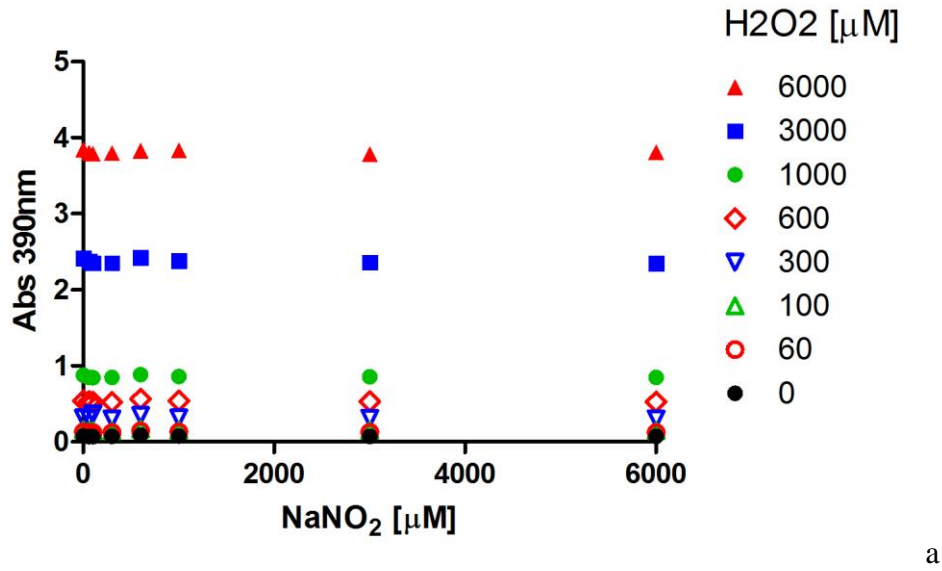


Figure 8a,b

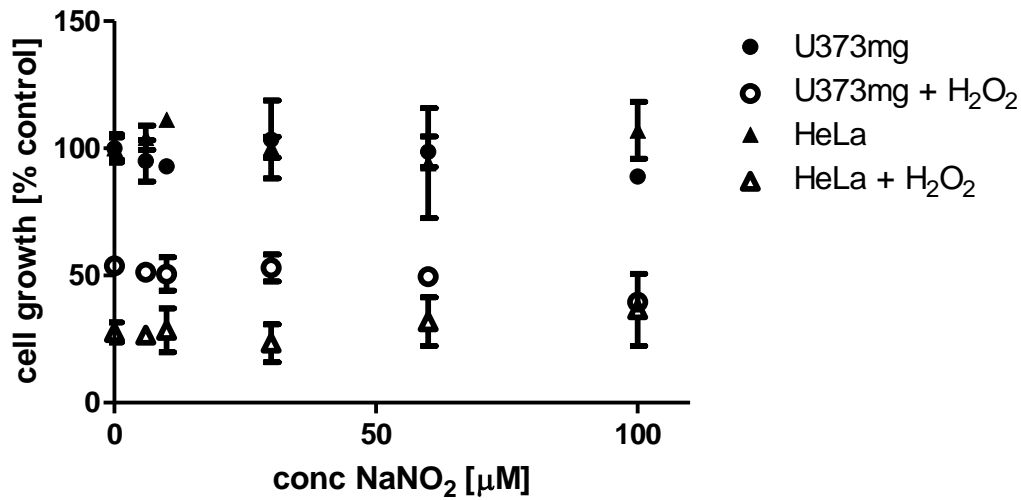
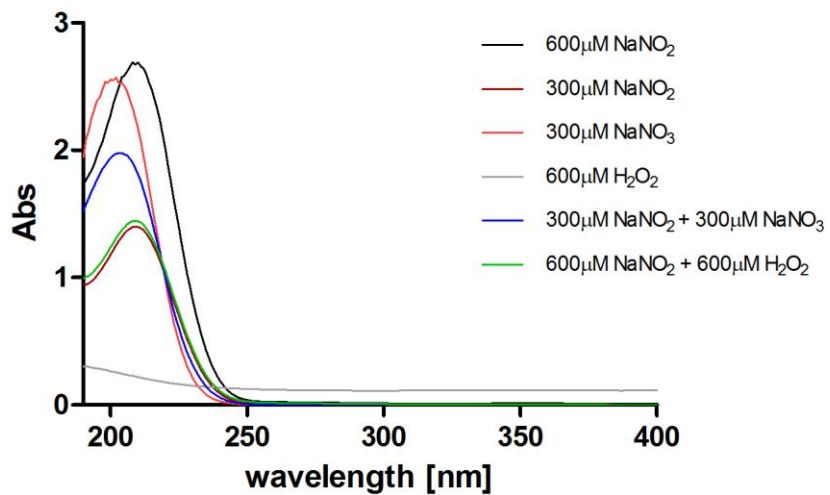
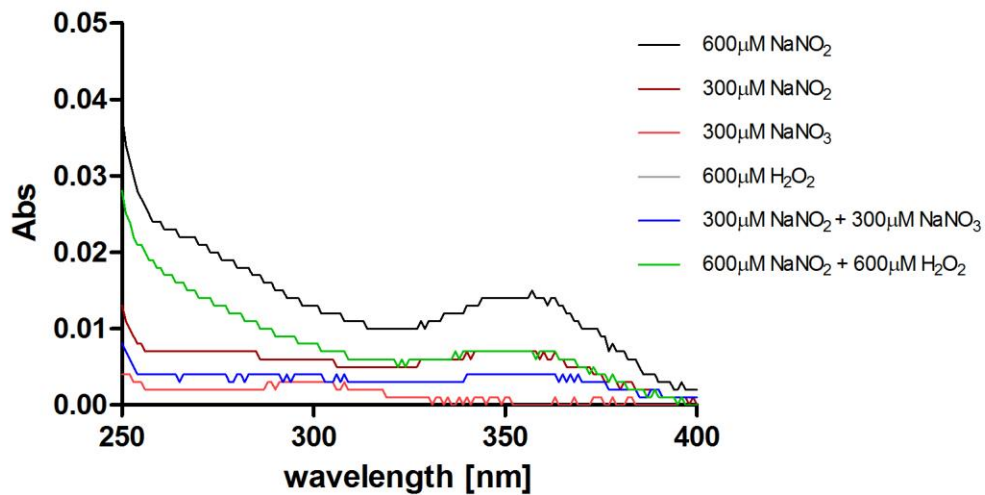


Figure 9



a



b

Figure 10a,b

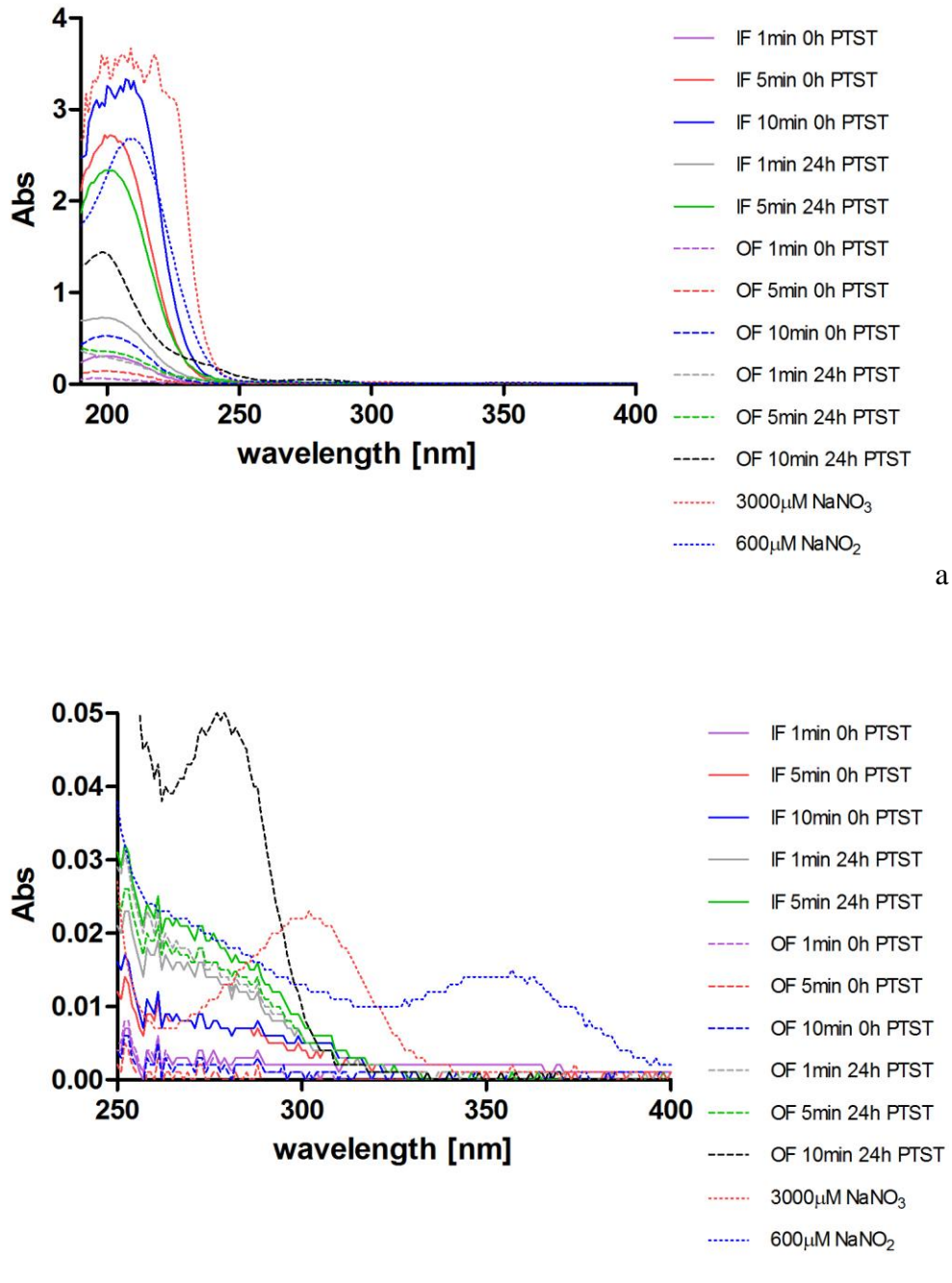


Figure 11a,b

**TABLES**

Table I: IC50 values (from curve fit) of cultures supplemented with pure hydrogen peroxide or PAW.

	IC50 H <sub>2</sub> O <sub>2</sub> (pure) [ $\mu$ M]	IC50 H <sub>2</sub> O <sub>2</sub> (PAW) [ $\mu$ M]
CHO-K1	225	105
HeLa	105	140
HaCaT	95	80
U373mg	315	185