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Alkaline plasma-activated water (PAW) as an innovative therapeutic avenue for cancer treatment

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ABSTRACT

Plasma-activated water (PAW) is considered to be an effective anticancer agent due to the diverse aqueous reactive oxygen and nitrogen species (RONS: ROS and RNS), but the drawback of low dose and short duration of RONS in acidified PAW limits their clinical application. Herein, this Letter presents an innovative therapeutic avenue for cancer treatment with highly-effective alkaline PAW prepared by air surface plasma. This anticancer alkaline formulation is comprised of a rich mixture of highly chemical RONS and exhibited a prolonged half-life compared to acidified PAW. The H_2O_2, NO_2^-, and $ONOO^-/O_2^-$ concentrations in the alkaline PAW can reach up to 18-, 16-, and 14-fold higher than that in acidic PAW, and the half-life of these species was extended over 8-, 10-, and 26-fold, respectively. The synergistic potent redox action between these RONS with alkaline pH was shown to be more potent than acidic PAW for cancer cell inhibition *in vitro*. Furthermore, the alkaline PAW injection treatment also significantly inhibited tumor growth in tumor-bearing mice. The possible reasons are that the alkaline PAW would disturb the acid extracellular milieu leading to the inhibition of tumor growth and progression; moreover, the efficient and durable RONS with alkaline pH could induce significant cell apoptosis by altering cell biomolecules and participating apoptosis-related signaling pathways. These findings offer promising applications for developing a strategy with real potential for tumor treatment in clinical applications.

Cancer is one of the most devastating conditions in the world, about 20×10^6 new cancer cases and 10×10^6 cancer deaths are reported each year.[1] The recent development in cancer therapy fueled significant interest in highly multidisciplinary approaches, tackling tumors on a much broader scale involving reactive oxygen and nitrogen species (RONS).[2,3] Plasma-activated solutions have been widely tested as promising anticancer RONS-generating methods and proved to induce the selective apoptosis of cancer cells.[4–6] Plasma-activated water (PAW) derived from the interaction of plasma with liquid (e.g., water or solutions) contains copious RONS, particularly long-lived species, such as NO_2^-, NO_3^-, H_2O_2, and O_3, and short-lived species, such as OH, O_2^-, and $ONOO^-$.[7–9] However, after plasma treatment, the activated solutions would become further acidified, and the anticancer species in acidic environments would quickly degrade during storage for several hours, which limits the PAW's clinical applications.[10]

Current studies have focused on the anticancer effect of RONS as the therapeutically active constituent but neglected the contribution of pH. The solution pH seriously affects the generation pathway of RONS[11–13] and then alters the effect of the application.[14] From the perspective of PAW chemistry, it has been reported that the self-degradation of NO_2^- and $ONOO^-$ in PAW would be significantly reduced in an alkaline environment.[15,16] From the clinical perspective of cancer treatment, the external pH of malignant cells (pH = 6.7) is slightly acidic compared to healthy cells (pH 7.2–7.5) due to the high production of lactic acid and CO_2 in metabolic processes, and acidity is essential for invasiveness, metastatic behavior, and resistance to

cytotoxic agents.[17,18] We propose that an efficient way to combat cancer could be to deprive the suitable environment of cells by correcting the acidity which is a critical living option and thus provoking tumor cell death.[19] Alkaline PAW injection could alkalify the cellular microenvironment of the tumor, and then the highly reactive RONS in PAW can induce significant apoptosis via related signaling pathways and biomolecules damage in cancer cells. Alkaline PAW has been found to display effective inactivation of microorganisms,[20] but its anticancer effect has not been reported elsewhere. The alkaline PAW may provide us with an innovative therapeutic avenue for cancer treatment.

In this study, the acid solution (HCl solution, pH = 3), neutral solution (deionized water, pH = 7), and alkaline solution (NaOH solution, pH = 12) were exposed to air surface plasma for different periods (1–6 min) to obtain acidic and alkaline PAW. The production and lifetime of RONS in acidic and alkaline PAW were evaluated at 5 min plasma treatment, and the corresponding anticancer effects on A549 cancer cells were conducted *in vitro*; furthermore, the inhibition of malignant tumors was validated in tumor-bearing mice. The research on the alkaline PAW could complement the theoretical basis for plasma liquid-phase chemistry and indicate that suppressing tumor acidity may represent promising antitumor strategies.

The pictorial overview of PAW preparation and physicochemical property detection were shown in Fig. 1(a). The air plasma device with a "sandwich structure" consist of a circular high-voltage (HV) copper (r = 60 mm) electrode, an FR-4 fiberglass dielectric substrate (a thickness of 1.5 mm), and a grounding mesh electrode (10 × 10 cm^2), which were integrated into a printed circuit board. The distance between the ground electrode and liquid level was 12 mm. Air plasma was obtained by applying a sinusoidal voltage (13 kV, 20 kHz) to the dielectric barrier discharge device. pH values of PAW were measured by a pH meter (METTLER-TOLEDO, FE20). Chemical fluorescent probes and a microplate reader (Varioskan Flash Reader; Thermo Fisher Scientific) were applied to measure concentrations of long-lived species, with a hydrogen peroxide assay kit (S0038; Beyotime) for H_2O_2 and a Griess reagent kit (S0024; Beyotime) for NO_2^- and NO_3^-. Concentrations of short-lived species were measured by an electron spin resonance spectrometer (BrukerBioSpin GmbH, EMX). The concentrations of $ONOO^-/O_2^-$ were reflected by the TEMPONE signals using the trap adduct (TEMPONE-H, Enzo). The total absorbance of PAW was obtained by a UV–visible spectrophotometer (Shimadzu U-1800) in the (200–300 nm) range to assess RONS delivery through the plasma to the liquid phase.[6] The A549 lung carcinoma cells were used to verify the anticancer effects by the mixture of 40% PAW + 60% medium *in vitro* and *in vivo*, the experimental details have been mentioned in the previous studies.[21,22]

To compare the activation effect of acidic and alkaline PAW, the performances of pH value, total absorbance, long-lived (H_2O_2, NO_2^-, and NO_3^-), and short-lived ($ONOO^-/O_2^-$) species in different initial pH solutions were shown in Fig. 1. The pH values reflected the

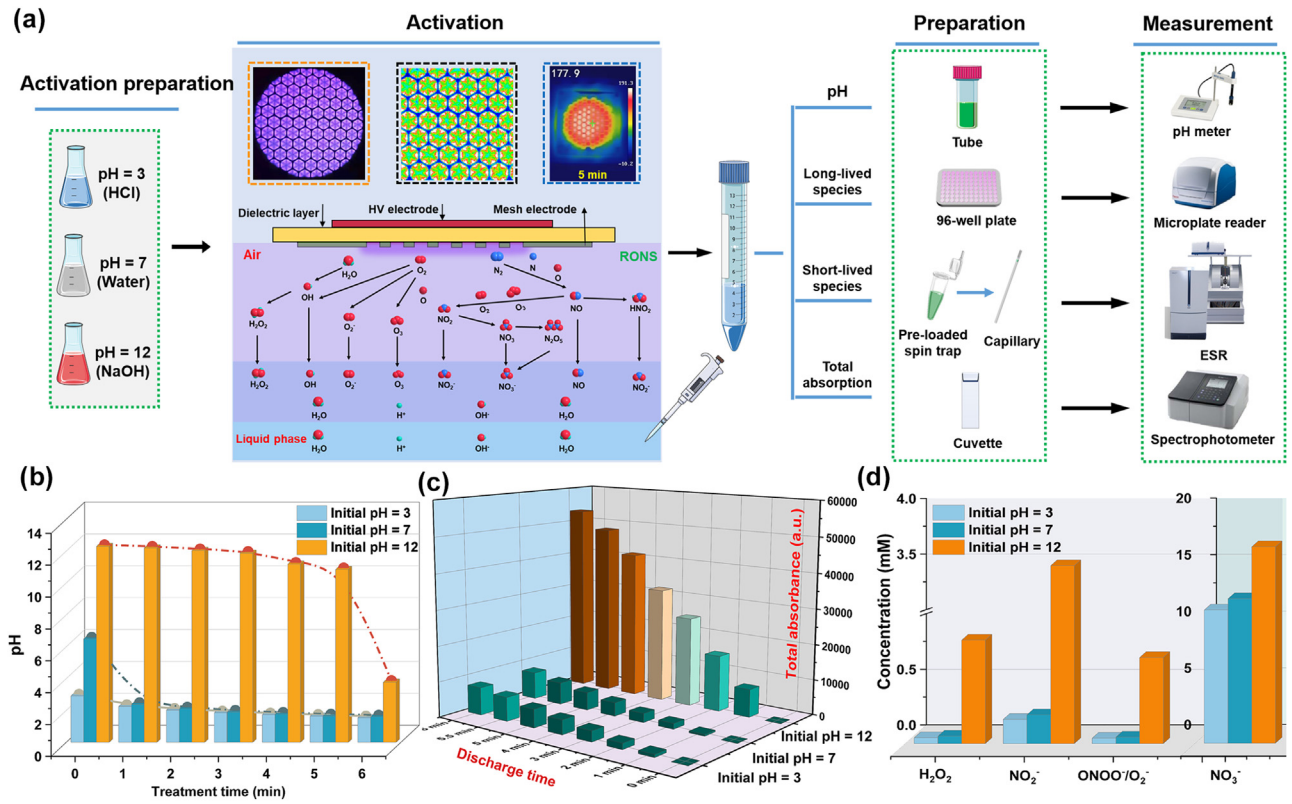

FIG. 1. (a) Sketch map of PAW preparation and physicochemical property detection; (b) the pH value as a function of the discharge time for different initial pH; (c) the total absorbance as a function of the discharge time for different initial pH; and (d) the RONS concentration after 5 min treatment for different initial pH.

acidification level of solutions over treatment time by the relationship $pH = -\log [H^+]$. As shown in Fig. 1(b), the pH value of the alkaline PAW continued to drop but with a much slower decay before 5 min and reached 10.85 and 3.78 at 5 and 6 min, respectively. The pH values of acidic and neutral initial solutions were reduced to below 3 over 1 min and dropped to 1.65 at 5 min plasma treatment. Concerning pH, the massive amount of H^+ ions produced during the sustained NO_x compounds (NO, NO_2, and N_2O_5) dissolution process leads to a continuous decrease in the pH of all PAW.[23] The RONS delivery effect through the air plasma to the solutions was evaluated by the total absorbance with discharge time,[24] and the result was shown in Fig. 1(c). The total absorbance values of PAW in alkaline conditions were consistently higher to other conditions, while the difference was not significant between the acidic and neutral conditions. This value for the alkaline situation is about 53 401 at 6 min, approximately seven times higher than others, which indicates that the alkaline condition was more favorable for the production of aqueous RONS. After 5 min plasma treatment, the alkaline solution remained high alkalinity (pH = 10.85), which was defined as the alkaline PAW, while the acidic and neutral solutions after treatment were defined as acidic PAW due to the same acidification (pH = 1.65). To compare the specific aqueous RONS concentration in acidic and alkaline PAW, the concentrations of H_2O_2, NO_2^-, NO_3^-, and $ONOO^-/O_2^-$ were detected after 5 min activation and the results were shown in Fig. 1(d). The concentrations of RONS in the acidic PAW group had no significant difference from each other. For the alkaline PAW, the values of H_2O_2 and NO_2^- concentrations were 0.9 and 3.56 mM, which were approximately 18 and 16 times higher than others. For short-lived species, the $ONOO^-/O_2^-$ concentration in the alkaline PAW (0.77 mM) was approximately 14 times as high as the acidic PAW. To assess the lifetime of these RONS, we tracked the change in concentrations at post-discharge times. As shown in Fig. 2, the half-life of H_2O_2 and NO_2^- in acidic PAW was about 21 and 16 h, which was estimated about 8 and 10 times lower than alkaline PAW (≥7 days). This prolonged activity of the alkaline PAW was also reflected in short-lived species. The $ONOO^-/O_2^-$ acquired an extended half-life of more than 8 h in the alkaline PAW far beyond the 0.3 h going to acidic PAW, realizing an enhancement of more than 2600%.

These results indicated that RONS in the alkaline PAW persists at higher levels and for a longer period than that in acidic PAW. The potential explanations will be discussed as follows. According to the present studies, the aqueous H_2O_2 mainly originates from two pathways: (1) gaseous H_2O_2 transformation and (2) the recombination of OH radicals at the gas/liquid interface,[7,25]

$$OH_g + OH_g \rightarrow H_2O_{2g} \rightarrow H_2O_{2aq}, \quad (1)$$

$$OH_{aq} + OH_{aq} \rightarrow H_2O_{2aq}. \quad (2)$$

The dissolution of gaseous H_2O_2 does not involve H^+ and OH^- in the liquid phase, so theoretically the dissolved H_2O_2 in the same amounts at all pH solutions. However, the removal reactions of O_3 are important for directly or indirectly providing the short-lived ROS under alkaline pH, namely,[12]

$$O_3 + OH^- \rightarrow O_2 + HO_2^-, \quad (3)$$

$$O_3 + OH^- \rightarrow O_2^- + HO_2, \quad (4)$$

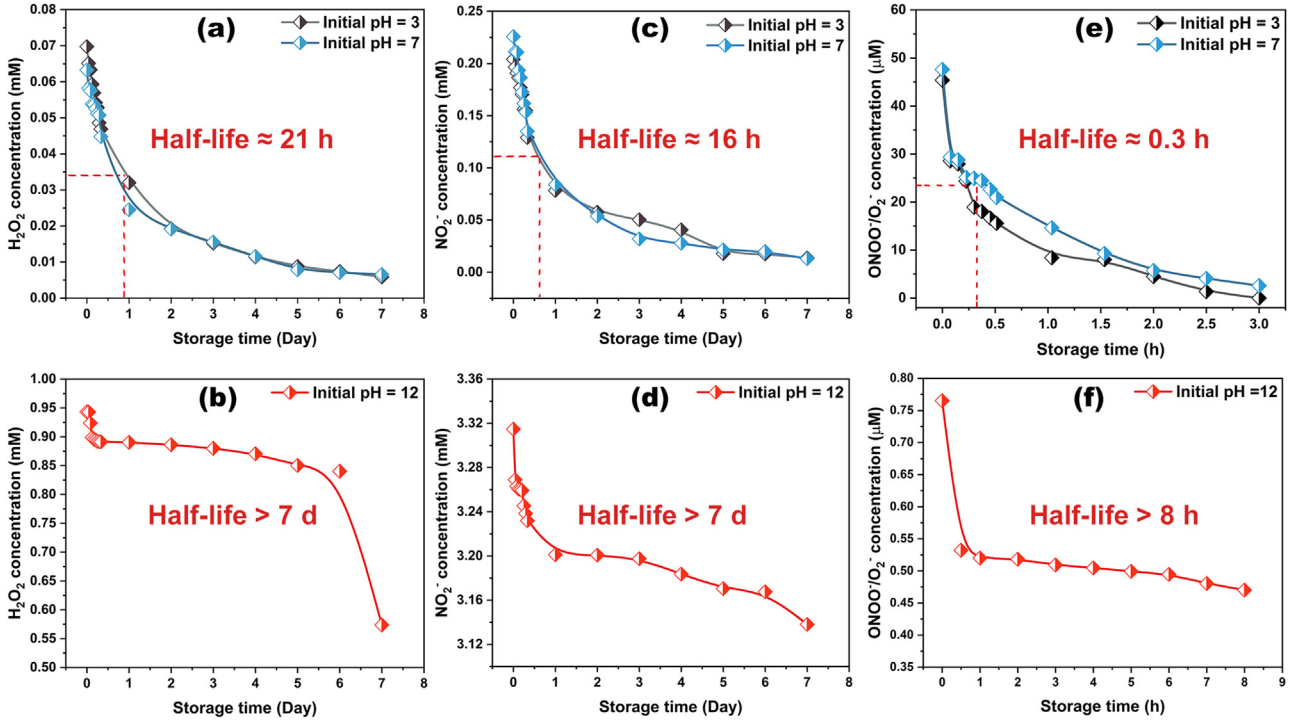

FIG. 2. The half-life of long-lived and short-lived species in the post-discharge time. The concentration of (a) and (b) H_2O_2, (c) and (d) NO_2^-, (e) and (f) $ONOO^-/O_2^-$ in PAW of different initial pH after 5 min plasma treatment as a function of storage time.

$$O_3 + HO_2^- \rightarrow O_2^- + O_2 + OH. \quad (5)$$

Thus, more aqueous H_2O_2 under the alkaline PAW is contributed by OH recombination from the removal of O_3. The aqueous NO_2^- and NO_3^- are derived from NO_x dissolution, and the generation efficiencies are substantially higher with an added base due to the HNO_2 deprotonation.[26] HNO_2 is in acid–base equilibrium (pKa = 3.3) with NO_2^-, the NO_2^- obtains domain concerns to HNO_2 in alkaline solutions which suppressed HNO_2 decomposition and its instantaneous reaction. The decomposition products of HNO_2 are NO and NO_2 via reaction (6).[27] NO_2 is further converted to NO_3^- as the final product by reactions (7) and (8), while NO continues the reaction cycle via reaction (9).[28] In addition, the consumption of HNO_2 is not only by reaction (10) but also by reaction (11) which reacts with H_2O_2 to generate ONOOH under acidic conditions

$$2HNO_2 \rightarrow NO + NO_2 + H_2O, \quad (6)$$

$$2NO_2 + H_2O \rightarrow NO_3^- + NO_2^- + 2H^+, \quad (7)$$

$$4NO_2 + O_2 + 2H_2O \rightarrow 4NO_3^- + 4H^+, \quad (8)$$

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+, \quad (9)$$

$$OH + HNO_2 \rightarrow H_2O + NO_2, \quad (10)$$

$$HNO_2 + H_2O_2 \rightarrow ONOOH + H_2O. \quad (11)$$

The reactivity of peroxynitrite is highly pH-dependent (pKa = 6.8), both anionic ($ONOO^-$) and protonated form (ONOOH) can involve in cellular redox reactions.[29] Since peroxynitrite formation could be ignored from the reaction (11) at pH $\geq$ 6.5, its formation would be through the reaction of NO and NO_2 at the gas–liquid interface via the following reactions:[13,30–32]

$$O_2^- + NO \leftrightarrow ONOO^-, \quad (12)$$

$$OH + NO_2 \rightarrow ONOOH, \quad (13)$$

$$O^- + NO_2 \leftrightarrow ONOO^-, \quad (14)$$

$$OH + NO_2 \leftrightarrow ONOOH. \quad (15)$$

The volatilization rate of NO_2 decreased from 88% to 19.5% at alkaline pH.[7] As the high O^- and O_2^- concentration supplied by the removal reaction of O_3 at alkaline conditions, abundant O^- could react with NO_2 to form $ONOO^-$ via reaction (14), with a total formation of $ONOO^-$ of 52%. Moreover, there are more decomposition routes of ONOOH under acidic conditions, namely, hemolysis (~30%) via reaction (16) and isomerization to nitric (~70%) via reaction (17),[8] but it would be ionized to stable $ONOO^-$ in alkaline solutions as the predominant form.[5] These reactions together contribute to the RONS concentration elevation and prolonged half-lifetime of the alkaline PAW

$$ONOOH \rightarrow OH + NO_2, \quad (16)$$

$$ONOOH \rightarrow H^+ + NO_3^-, \quad (17)$$

$$ONOO^- \rightarrow O_2^- + NO. \quad (18)$$

To investigate the inhibition effect of different PAW against tumor cells *in vitro*, the viability of A549 cells was shown in Fig. 3. It should be mentioned that the pH value of the untreated solutions in the control group was consistent with the corresponding PAW. A minor difference in the control treatment group can be observed, which indicates that the pure pH had a limited effect on cell viability. The death rate exhibited an insignificant difference with an initial pH of 3 and 7, while the death rate was 91.59% for the initial solution pH = 12 indicating that the alkaline PAW can effectively inhibit the viability of A549 cells more than others. This result was corroborated by microscopic image, alkaline PAW treatment induced the highest number of trypan blue-positive cells (dead cells) of all. Furthermore, *in vivo* tumor cell proliferation inhibition evaluations were assessed by *in vivo* fluorescence imaging of the tumor-bearing mice after PAW

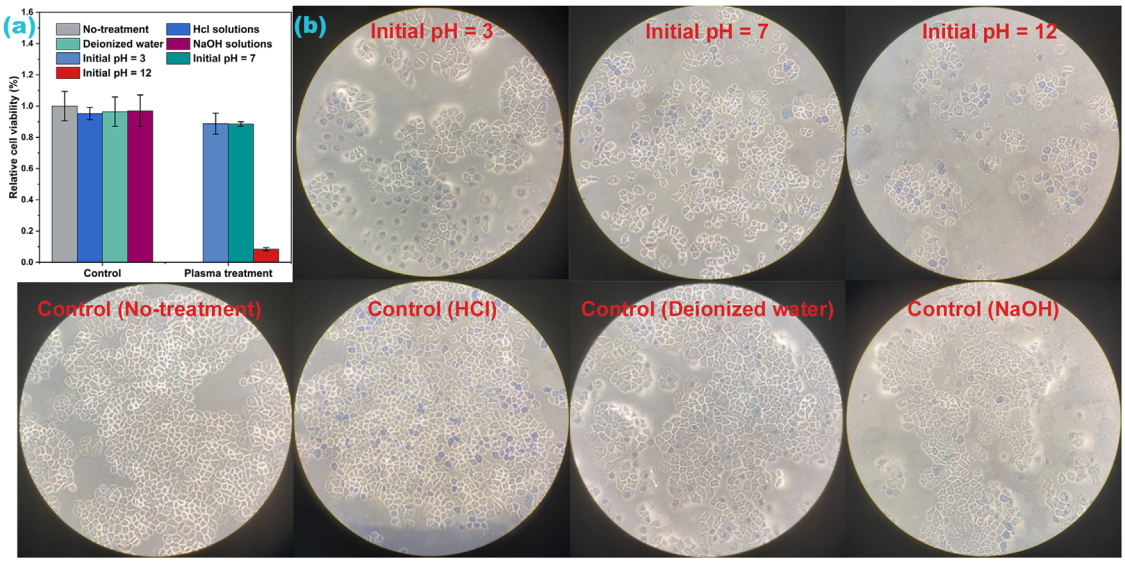

FIG. 3. *In vitro* anticancer effects of plasma-activated water under different initial pH after 5 min plasma treatment. (a) The viability of A549 cancer cells treated with different plasma-activated water and (b) the colored images obtained using an inverted microscope (the dead cells are stained blue). The student's t-test was applied to analyze the statistical significance. One $p < 0.05$ between two independent groups was considered to indicate statistical significance ($^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

injection, and the results were shown in Fig. 4. After 14 days of treatment, the tumors with the alkaline PAW injection underwent no distant metastasis and the bioluminescence areas of the tumors analyzed by ImageJ software were about 1.7 times smaller than that without the PAW treatment which significantly inhibited tumor growth. The inhibition effect of the alkaline PAW might provide a good therapeutic approach given its tumor-suppressive role.

The alkaline PAW may block the growth and function of the cancer cells in two main ways. On the one hand, the alkaline PAW deprives the mildly acidic environment of cells by correcting the pH which is a critical living option. This could disrupt the extracellular acidity homeostasis of cancer cells, thus limiting cancer growth and progression by the reduction in matrix degradation (cathepsin and metalloproteinases).[33] The elevation of extracellular pH could also improve immunological defenses and increase tumor access to anticancer species *in vivo*.[18,34] On the other hand, the alkaline PAW possesses lasting and high concentration oxidative ROS and RNS: H_2O_2 and peroxynitrite, both of which and their secondary species (such as OH, O_2^-, NO, $^1O_2$) with alkaline pH may involve in cell apoptosis. And the disruption of the microenvironment renders cancer cells more vulnerable to extrinsic oxidative stress by aqueous RONS.[35] H_2O_2 is known to be able to pass through the cell membrane via aquaporins, which are more highly expressed in malignant cells than in normal cells, allowing more ROS to through the cell membrane and induce apoptosis via oxidative stress.[36] The decomposition of peroxynitrite results in the generation of $^1O_2$ and triggers an autoamplificatory process, in which malignant cells, but not nonmalignant cells, are contributing to their cell death through the promotion of secondary $^1O_2$ generation, catalase inactivation, and reactivation of intercellular apoptosis-inducing ROS signaling.[37] Overall these high levels of RONS could not only cause direct alteration to biomolecules (membrane lipid peroxidation, protein modification, and DNA damage) which involves initiating induced apoptosis but also taking part in several apoptosis-related signaling pathways, such as mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways.[38] The synergistic effect of these factors finally leads to cell death by selective apoptosis.

Overall, this study demonstrates that the alkaline PAW possesses a superior anticancer effect to acidic PAW. One of the major advantages of the alkaline PAW is the ability to produce high-yield RONS with a long shelf-life without the requirement of refrigeration. Compared with the acidic PAW, the concentrations of H_2O_2, NO_2^-, and $ONOO^-/O_2^-$ in the alkaline PAW were enhanced by 18, 16, and 14 times and the half-life of those were prolonged by 8, 10, and 26

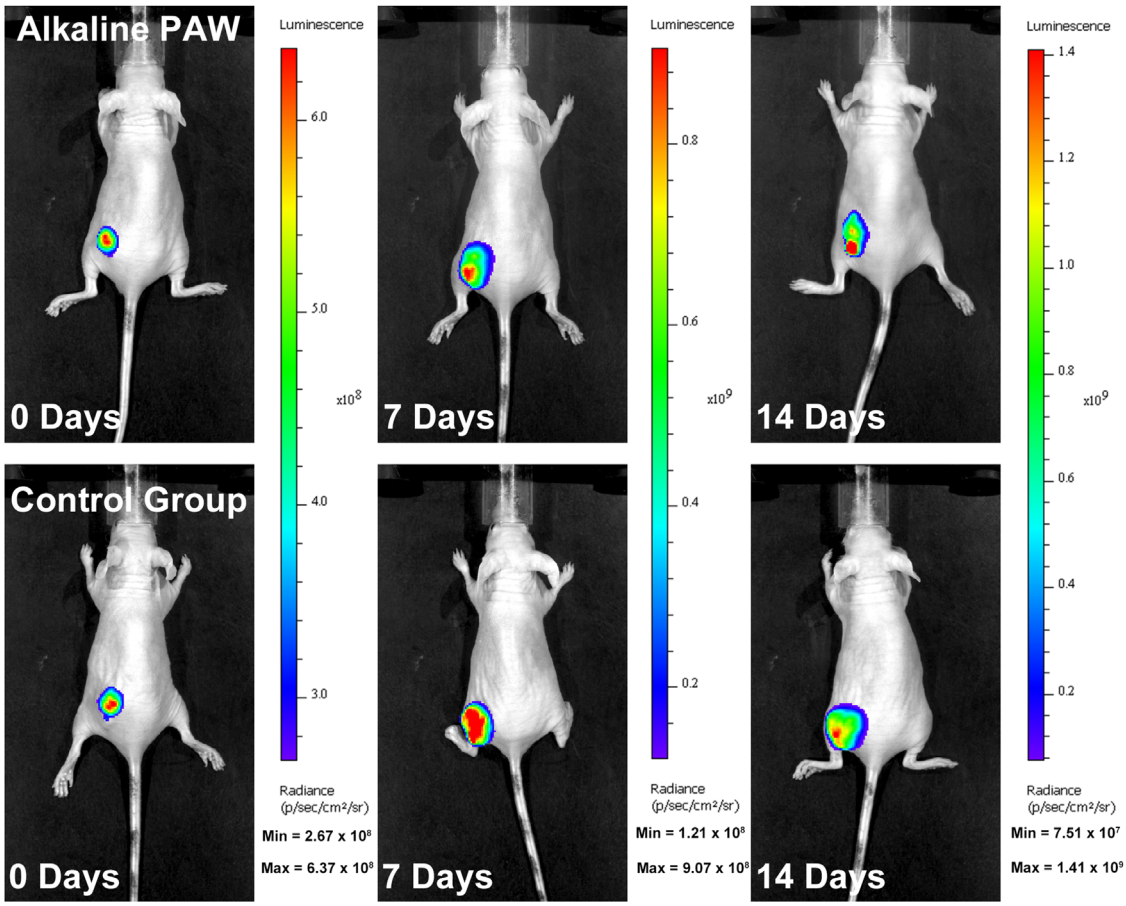

FIG. 4. The growth of tumor shown by a whole-body fluorescent imaging system after the alkaline PAW treatment.

times at least. Moreover, the alkaline PAW could induce significant apoptosis of A549 cancer cells *in vitro* compared to other conditions and it could further inhibit tumor growth *in vivo*, which attribute to the combination of RONS and alkaline microenvironment pH. This research not only complements the theoretical basis for plasma liquid-phase chemistry but also elucidates that alkaline PAW can potentially be utilized as a promising therapeutic in tumor treatment.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Ethics Approval

The study was approved by the laboratory animal care committee of Xi'an Jiaotong University (No. XJTULAC2020–215) and was performed according to the committee's guidelines for the use of laboratory animals.

Author Contributions

Bolun Pang: Conceptualization (lead); Methodology (lead); Writing – original draft (lead); Writing – review & editing (equal). **Zhijie Liu:** Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Investigation (equal); Methodology (supporting); Project administration (equal); Supervision (equal); Visualization (supporting); Writing – original draft (supporting); Writing – review & editing (equal). **Sitao Wang:** Investigation (equal). **Yuting Gao:** Investigation (supporting). **Miao Qi:** Methodology (supporting). **Dehui Xu:** Methodology (supporting). **Renwu Zhou:** Data curation (supporting); Formal analysis (supporting); Project administration (supporting). **Dingxin Liu:** Supervision (equal). **Michael G. Kong:** Supervision (equal).

DATA AVAILABILITY

The data that support the findings of this study are available within the article.

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