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Targeted hyperalkalization with NaOH-loaded starch implants enhances doxorubicin efficacy in tumor treatment

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ABSTRACT

High-alkali treatment using sodium hydroxide (NaOH) injection can be a therapeutic approach for killing tumor cells. Alkalization can damage cellular structures and lead to cell death. Increased alkalinity can also enhance the efficacy of certain chemotherapeutic drugs such as doxorubicin (DOX). In this study, NaOH-loaded starch implants (NST implants) were used to induce hyperalkalization (increase pH) in the tumor environment, thereby inducing necrosis and enhancing the effects of DOX. NaOH is a strongly alkaline substance that can increase the pH when injected into a tumor. However, the administration of NaOH can have toxic side effects because it increases the pH of the entire body, not just at the tumor site. To overcome this problem, we developed an injectable NST implant, in which NaOH can be delivered directly into the tumor. This study showed that NST implants could be easily administered intratumorally in mice bearing 4T1 tumors and that most of the NaOH released from the NST implants was delivered to the tumors. Although some NaOH from NST implants can be systemically absorbed, it is neutralized by the body's buffering effect, thereby reducing the risk of toxicity. This study also confirmed both *in vitro* and *in vivo* that DOX is more effective at killing 4T1 cells when alkalized. It has been shown that administration of DOX after injection of an NST implant can kill most tumors. Systemic absorption and side effects can be reduced using an NST implant to deliver NaOH to the tumor. In addition, alkalinization induced by NST implants not only exerts anticancer effects but can also enhance the effect of DOX in killing cancer cells. Therefore, the combination of NaOH-loaded starch implants and DOX treatment has the potential to be a novel therapy for tumors.

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1. Introduction

Extracellular pH of tumor cells is more acidic than that of normal cells. The extracellular pH around tumor cells typically ranges from 6.4 to 7.0, while the extracellular pH of normal cells is generally within the range of 7.2 to 7.5 [1,2]. This difference in pH is due to the Warburg effect, a phenomenon in which cancer cells produce lactate through anaerobic glycolysis even when sufficient oxygen is available [3,4]. Lactate production and poor tumor perfusion result in the acidification of the extracellular tumor environment. The low pH of tumors can negatively affect cancer treatment and progression. For example, it can lead to drug resistance, as some anticancer drugs are less effective at low pH. It can also promote the spread of cancer cells (metastasis) and support the growth of new blood vessels (angiogenesis) that feed the tumor, making it harder to treat [5,6]. Therefore, the acidic pH of tumors is a target for cancer treatment, and several strategies are being developed to increase the pH and improve the effectiveness of anticancer therapies.

Studies have shown that controlling the acidity of the tumor microenvironment (TME) through alkalinization can enhance antitumor effects. Sodium bicarbonate is an alkaline substance that has been extensively studied for this purpose [7–9]. Electrochemical therapy (EChT) is also a more direct method of controlling the tumor's microenvironment. It involves the application of a direct current between multiple electrodes, which generates both acids and bases in the tumor. At the cathode, a high concentration of hydroxide ions is generated, which can cause tumor necrosis by creating an alkaline environment in the tumor [10–12].

NaOH is a strong, highly corrosive alkali that can cause cell necrosis. Highly alkaline conditions lead to the inactivation or denaturation of enzymes and structural proteins; cells swell and break down, resulting in necrosis [13,14]. However, NaOH solution injected directly into the body not only causes necrosis at the injection site, but is also quickly absorbed throughout the body and causes alkalinization of the whole body, which can lead to toxicity. In this study, NST implants were prepared to reduce the systemic absorption of NaOH and to deliver it to the administration site.

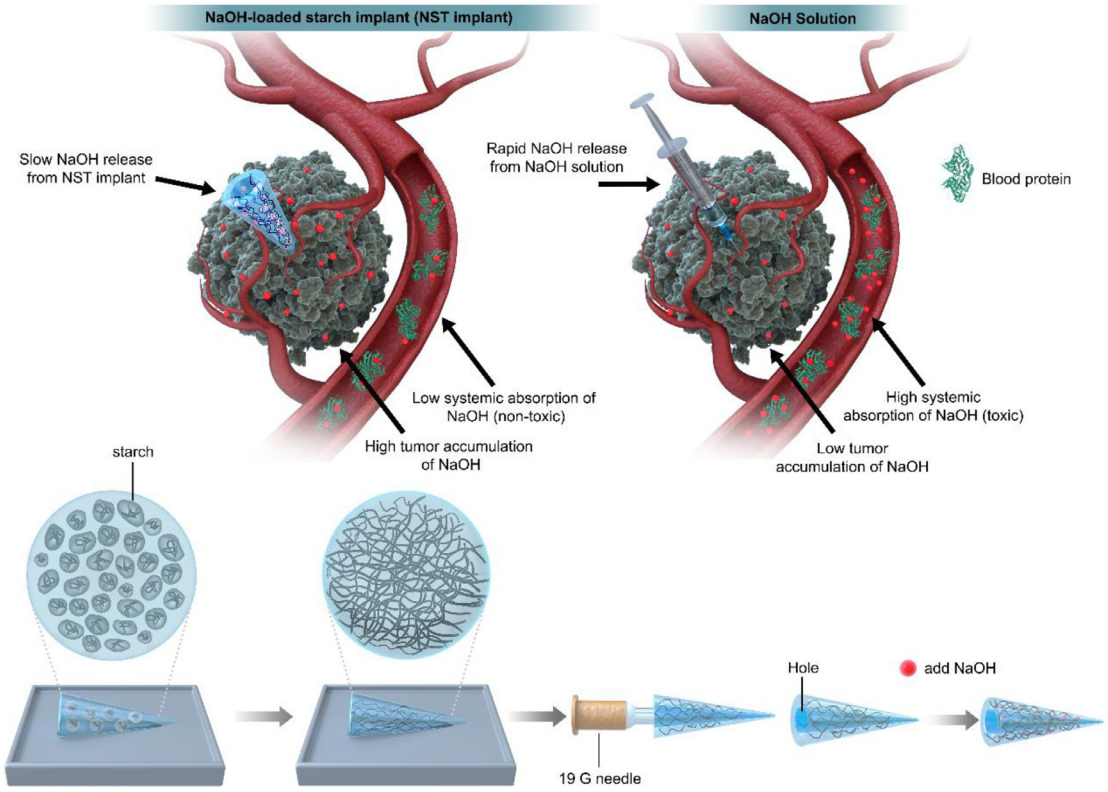
Starch is the second most abundant biopolymer on Earth, after cellulose chitin, and is cheap and readily available. Starch can be used in a variety of biomedical materials including drug delivery systems due to its biodegradability, biocompatibility and low immunogenicity [15–17]. When heat disrupts the crystalline structure of starch and leads to gelatinization of the starch solution, it can absorb moisture to generate a stable three-dimensional hydrogel network. As a result of retrogradation during the drying process, starch recrystallizes, and the resulting starch implants become rigid enough for injection [18,19]. Furthermore, the adaptability of starch implants in terms of shape and size allows precise administration to the target site. Although microneedles facilitate easy and painless administration, their short length limits effective drug delivery to the surface of the tumor [20]. However, starch implants can be tailored to tumor size, which enables them to function as needle-type implants facilitating effective drug delivery throughout the tumor. These characteristics allow starch implants to deliver drugs within tumors more uniformly and effectively than microneedles, solutions, or hydrogels [15].

Because NaOH can degrade starch, it was added after the creation of starch implants [21]. After manufacturing the starch implant with a hole, NaOH was placed in the hole of the starch implant (NST implant) and dried so that NaOH could be absorbed by the implant. Because NaOH is dried, it does not degrade the starch needles. Because the NaOH in the NST implant is dried in the implant, it is released from the body more slowly than the NaOH solution due to the time it takes to dissolve. NaOH causes acid-base reactions with acidic substances in the body, including tissues, proteins, and other components [22]. In particular, tumors have a dense structure and are composed of various acidic substances [23,24]. Because the acid-base reaction is very fast, the NaOH released from the NST implant reacts immediately with acidic substances inside the tumor, avoiding systemic absorption. This indicates that the NST implant can deliver NaOH to the tumor more effectively than systemic absorption, depending on the delayed release rate.

In addition, the body has a strong buffering effect. The bicarbonate buffer system is one of the most important buffer systems in the body, helping to maintain a stable pH in the blood [23,25,26]. The bicarbonate buffer system can rapidly neutralize excess hydrogen or hydroxide ions in the blood by regulating the carbon dioxide in the body through breathing. When there is an excess of hydroxide ions in the blood (alkaline environment), the body can reduce the rate and depth of breathing, allowing it to retain more CO_2 [27]. This increases the concentration of carbonic acid in the blood and produces more bicarbonate ions to neutralize excess hydroxide ions, thereby reducing the blood pH. Most proteins in the body act as buffers. Proteins are composed of amino acids that contain positively charged amino groups and negatively charged carboxyl groups, and can act as buffers by combining with hydrogen and hydroxide ions [28,29]. The buffer system of the human body is highly effective and can adjust pH within seconds. Therefore, the small amount of NaOH absorbed from the NST implant into the body is neutralized by the body's buffer system and is nontoxic.

In addition, tumor alkalinization by NST implants can generate synergistic effects with chemotherapeutic agents, such as doxorubicin (DOX). DOX, an ionizable weakly basic drug, can freely pass through cell membranes in its uncharged form. However, in an acidic tumor environment, DOX, an ionizable weakly basic drug, becomes charged, which inhibits its permeability through cell membranes. This can lead to a reduction in cellular uptake and potency [30–32]. NST implants can enhance the therapeutic effect of DOX by alkalizing the acidic environment of tumors.

This study demonstrated that the NST implant could efficiently deliver NaOH to the tumor by controlling the release rate of NaOH. Moreover, NST implants can induce tumor necrosis through alkalinization and offer a new treatment approach with synergistic effects when combined with DOX (Scheme 1).



Scheme 1 – Scheme of NaOH-loaded starch implant (NST implant) that can effectively deliver NaOH to tumors.

2. Materials and methods

2.1. *Materials*

Potato starch was purchased from Daejung Chemicals (Gyeonggi-do, South Korea). Gelatin was purchased from Sigma (St. Louis, MO, USA). 4T1 cells were purchased from the American Type Culture Collection. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Dublin, Ireland). All other reagents were obtained from Sigma-Aldrich unless otherwise specified.

BALB/c and ICR mice (male, 6 weeks old) were purchased from Orient Bio. All the mice were maintained under specific pathogen-free conditions. All *in vivo* experiments were performed in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee of Dongguk University, Gyeongju, Korea. All animal experiments were approved by the Animal Ethics Committee of dongguk (Gyeongju) University

2.2. *Preparation of NaOH-loaded starch implant (NST implant)*

The NST implant was prepared by modifying a previously described method [15,16]. Potato starch (300 mg) was mixed with 400 μl water and poured into a silicone mold. After incubating the mixture for 20 min at 80 °C for gelatinization, a 19 G needle was inserted into the center of the implant and dried for 1 d at 25 °C. After drying, the 19 G needle was removed to fabricate starch implants with a central hole. NST implants were prepared by adding 3 μl distilled water (DW) and 5 M, 10 M or 20 M NaOH solution per starch implant (ST implant, 2-NST implant, 10-NST implant, 20-NST implant) and drying them for 1 d at 25 °C.

2.3. *Characterization of NST implant*

The 20-NST implants were photographed to measure size and shape. To confirm delayed NaOH release, the 20-NST implant or 3 μl of 20 M NaOH solution were immersed in 10 ml of 10 mM phosphate-buffered saline (PBS) (pH 7.4) and PBS (pH 7.4) with 40 mg/ml of albumin, respectively, and pH was measured at predetermined time points (0, 5, 10, 20, 30, 40, 50, 60, 80. 100, 120, 140, 160 and 180 s) using a pH meter. The morphologies of ST, 10-NST and 20-NST implants were analyzed using scanning electron microscopy (SEM). The 20-NST implant was injected into the dissected tumor, confirming that it could be injected into the tumor without special equipment.

2.4. *Post-injection retention of Cy5-NST implant in the liver*

Livers obtained from dissected mice were used to verify the post-implantation retention of the NST implants. An NST implant loaded with Cy5 (Cy5-NST implant) was employed. Potato starch (100 mg) was mixed with 400 μl water followed by the addition of Cy5-carboxylic acid. Subsequently, the NST

implant preparation (described in Section 2.2) procedure was followed. Next, the Cy5-NST implant was injected into the liver, and its retention was monitored using a fluorescence device at specific intervals (0, 1 and 4 h).

2.5. *In vitro cytotoxicity*

The toxicity of NaOH, DOX and NST implants was evaluated using the MTT assay. The 4T1 cells were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS [33]. First, to evaluate the toxicity according to the concentration of NaOH and DOX, cells (10^5 cells/ml) were cultured in a 96-well plate for 24 h (100 µl/well). Subsequently, defined amounts of DOX (0, 1, 2, 4, 8 µg/ml) and NaOH (0, 2, 10, 20 mM) were added to the cells, which were incubated for 24 h. After treatment incubation, cytotoxicity was assessed using the MTT assay. To evaluate the toxicity of the NST implant and DOX, the 4T1 cells (10^5 cells/ml) were cultured for 24 h in a 6-well plate (2 ml/well). (I) ST implant, (II) 2-NST implant, (III) 10-NST implant, (IV) 20-NST implant, (V) DOX, (VI) combination of 20-NST implant and DOX (DOX 4 µg/ml) were added to the cells and cultured for 24 h. After 24 h, cytotoxicity was assessed using the MTT assay. To avoid inactivation of DOX in an alkaline environment, DOX was administered 30 min after 20-NST implant administration in the (VI) combination of 20-NST implant and DOX

2.6. *In vitro cell pH*

To measure the pH change in the cell culture medium after NST implantation, the 4T1 cells (10^5 cells/ml) were cultured for 24 h in a 6-well plate (2 ml/well). After cell culturing, (I) 2-NST, (II) 10-NST, (III) 20-NST implants, (IV) DOX, and (V) combination of 20-NST and DOX (DOX 4 µg/ml) were added to the cells. The pH of the cell culture medium was measured at a specific time (0, 1, 4, 8, 24 h) using a pH meter, and photographs were taken.

2.7. *Cellular uptake of DOX at different pH*

The intracellular uptake of DOX at different pH was confirmed using confocal microscopy. Initially, 4T1 cells (10^5 cells/ml) were cultured in an 8-well chamber for 24 h. The medium was then replaced with a medium of pH 8.0, 7.4, 6.8 or 6.2, and DOX was added to achieve its final concentration of 4 µg/ml. Cells were washed with PBS after 4 h of treatment. DOX fluorescence was analyzed at 485/600 nm (excitation/emission wavelengths). The cells were imaged using a confocal microscope (LSM 800; Zeiss, Germany).

2.8. *In vivo toxicity and tumor necrosis of 20-NST implant and NaOH solution*

The toxicities of the 20-NST implant and NaOH solution were evaluated in BALB/c mice. Then, 3 µl 20 M NaOH solution or 20-NST implants were subcutaneously injected into the mice. After injection, the mice were observed and photographed for 6 weeks. In addition, tumor necrosis by 20-NST implant administration was confirmed. For this purpose, 4T1 cells were injected into the flank of the mouse. Subsequently, the 20-NST implant was injected into the tumor when its size reached 200–300 mm^3; the mice were observed and photographed before and after the treatment.

2.9. *Distribution of NaOH in gelatin/agar/albumin gel injected with NST implant*

A gelatin/agar/albumin gel was used to check the distribution of NaOH in the NST implant. First, an agar solution was prepared by adding 1.5 g agar to 50 ml 10 mM PBS (pH 7.4) and stirring at 100 °C for 30 min. Separately, a gelatin solution was prepared by dissolving 4 g gelatin in 200 ml 10 mM PBS (pH 7.4) at 37 °C for 30 min. Gelatin/albumin solution-1 was prepared by dissolving 2 g albumin in 100 ml the gelatin solution, followed by the addition of 500 µl universal indicator and 80 mg mTG. Gelatin/albumin solution-4 was prepared by dissolving 8 g albumin, 500 µl universal indicator, and 80 mg mTG in 100 ml the gelatin solution.

Next, after mixing 50 ml gelatin/albumin solution-1 or gelatin/albumin solution-4 with 50 ml agar solution, respectively, pour 10 ml the resulting mixture into a silicone mold to create gelatin/agar/albumin gel-1 (albumin concentration: 10 mg/ml) or gelatin/agar/albumin gel-4 (albumin concentration: 40 mg/ml). The gels were stored at room temperature for 1 d and used the next day.

Gelatin/albumin/agar gel were incubated in 10 ml 10 mM PBS (pH 7.4) containing both 100 mg albumin and 50 µl universal indicator, while gelatin/agar/albumin gel-4 were incubated in 10 ml 10 mM PBS (pH 7.4) containing both 400 mg albumin and 50 µl universal indicator at 37 °C. (I) 2-NST, (II) 10-NST, (III) 20-NST implants, and (IV) 3 µl NaOH solution were injected into gelatin/agar/albumin gel-1 or gelatin/agar/albumin gel-4. And the gels were observed at set times (0, 1, 2, 4, 8 h).

2.10. *20-NST implant-induced alteration in pH of blood and tumor*

To explore the effects of the 20-NST implant on the pH of the TME, 4T1 cells (1×10^6 cells) were injected into the flanks of the mice. When the tumor size reached 200–300 mm^3, the mice were treated with the 20-NST implant. Using a needle-type pH meter (Eutech^{TM} pHSpear pH Meter), we measured the temporal change in pH in three regions: the tumor core, the tumor periphery, and normal tissue (2 cm away from the tumor). Additionally, the pH of the blood samples, collected from the tail vein, was measured at set intervals using a pH instrument (LAQUAtwin pH-11).

2.11. *In vivo tumor therapy*

To evaluate the anticancer effect of the 20-NST implant, DOX and the combination of 20-NST implant and DOX, 4T1 cells (1×10^6 cells) were injected into the flanks of mice. Mice were treated through the intratumoral administration of (I) PBS, (II) ST-implant (III) 20-NST implants, (IV) DOX (2.5 mg/kg), (V) combination of 20-NST implants and DOX (2.5 mg/kg) when the tumor size reached 200–300 mm^3. In the combination of 20-NST implants and DOX, to avoid inactivation of DOX in an alkaline environment, DOX was administered intratumorally

30 min after 20-NST implant administration. The tumor size was measured every 2 d, On Day 16, the mice were euthanized, and the tumors were dissected and photographed.

2.12. *Hematoxylin and eosin (H&E) staining of organ*

ICR mice were treated with (I) Cotnrol, (II) 20-NST implant, (III) combination of 20-NST implant and DOX (2.5 mg/kg), and after 14 d, the major organs were dissected and collected ($n = 3$). H&E staining of the organs was performed according to the manufacturer's protocol, and the results were evaluated using a digital microscope.

2.13. *Hematology, biochemical analysis*

For hematological and biochemical analyses, ICR mice were treated with PBS (control) or a combination of 20-NST implant and DOX. Two weeks post-treatment, 300 µl blood was collected and placed in EDTA tubes for hematological analysis; several parameters, including white blood cell (WBC) count, red blood cell (RBC) count, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), hemoglobin (HGB), platelets (PLT) and mean corpuscular hemoglobin (MCH), were measured. For biochemical analysis, 600 µl blood was collected in the SST tube and allowed to coagulate at room temperature for 1 h; subsequently, it was centrifuged at 7000 rpm for 5 min to obtain 200 µl serum, which was subsequently used for biochemical analysis. The biochemical parameters included: aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), blood urea nitrogen (BUN), creatinine (CREA), globulin (GLB), and total bilirubin (TB), were analyzed.

2.14. *TUNEL assay and H&E staining of tumor*

When tumors in BALB/c mice were 200–300 mm^3, they were treated with the (I) 20-NST implant, (II) DOX (2.5 mg/kg), (III) ST implant, (IV) combination of 20-NST implant and DOX (2.5 mg/kg). After 2-d treatment, the tumors were dissected and collected. H&E staining and TUNEL assays were performed according to the manufacturer's protocol. The results were then observed under a microscope.

2.15. *Statistical analysis*

All the statistical analyses were performed using Microsoft Excel. Data were analyzed using the two-sample Student's *t*-test. Differences were considered significant at $P < 0.05$ (indicated by an asterisk in the figures).

3. Result and discussion

3.1. *Preparation and characteristics of NST implant*

In the present study, injectable NST implant were manufactured using silicone mold. The ST implant were 2 mm thick and 9 mm long, and were created with a hole in the center using a 19 G needle (Fig. 1A). 3 µl NaOH solution (2 M, 10 M or 20 M per implant) was absorbed into the starch implant through the hole and dried. To confirm the slow release of NaOH from the NST implant, PBS (pH 7.4) alone and PBS with 40 mg/ml albumin (PBS and alb-PBS) were used. First, when 3 µl of a 20 M NaOH solution was added to PBS and alb-PBS, the pH immediately changed. When 20-NST implants were added to PBS and alb-PBS, the pH changed slowly over a period of 3 min. The 20-NST implants showed smaller and slower changes in pH in alb-PBS than in PBS. (Fig. 1C and 1D). These results showed that NaOH exhibited a delayed release from the NST implant. They also show that biological proteins, such as albumin, can neutralize pH changes via a buffering effect.

It was confirmed that the NST implant had sufficient rigidity to be injected in the form of a needle; therefore, it could be easily injected into the tumor without special equipment (Fig. 1B). The drug delivery system, when directly administered, must remain at the site of administration. A dissected mouse liver was used as an *ex vivo* model to confirm the post-administration stability of the implant at

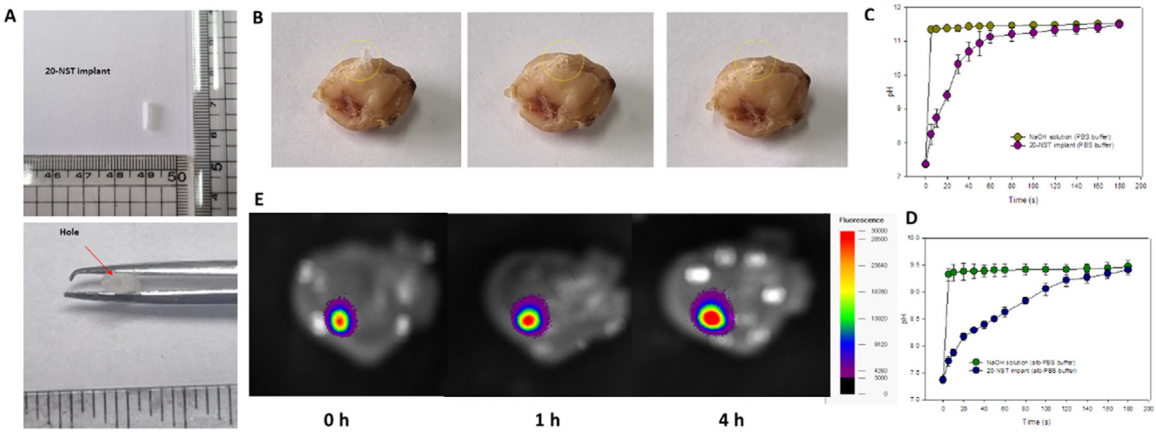


Fig. 1 – Photographs of (A) 20-NST implant and (B) 20-NST implant injection into the dissected tumors. pH change of NaOH solution and 20-NST implant in (C) PBS and (D) alb-PBS ($n = 3$) (E) Fluorescence images of the dissected liver at different time after Cy5-NST implant injection.

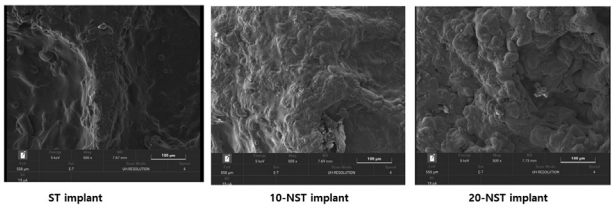


Fig. 2 – SEM images of ST implant, 10-NST implant, and 20-NST implant.

the site of injection. After the Cy5-NST implant was injected, the fluorescence confirmed that it remained in the liver for 4 h (Fig. 1E). Furthermore, the gradual release of Cy5 at the injection site was noticed. These findings suggested that the Cy5-NST implant can persist at the injection site for extended periods and effectively deliver the encapsulated drug to the surrounding region

The structures of the NST implants were confirmed by SEM. NST implant gelatinize during heating and recrystallize via retrogradation during drying. NST implants produced by this process exhibited a dense morphology. Even when the implants were filled with 10 M and 20 M NaOH, the structure of the starch implants did not show any difference (Fig. 2). This result confirms that NaOH loading does not affect the structure of the NST implants.

3.2. *In vitro cytotoxicity*

The cytotoxicity and synergistic effect of NaOH and DOX were evaluated through the MTT assay. When treated with NaOH alone, little cytotoxicity was observed at a concentration of 10 mM, and when treated with 20 mM NaOH, significant cytotoxicity was observed. The therapeutic effect of DOX was proportional to its concentration. It was confirmed that the effect of DOX greatly increased when used in combination with NaOH. For example, when treated separately, 10 mM NaOH and 2 μg DOX showed little cytotoxicity, but when combined, they showed significant toxicity (Fig. 3A, viability; only 10 mM NaOH: 91.37%, only 2 μg DOX: 83.34%, combination of 10 mM NaOH and 2 μg DOX: 47.12%).

Additionally, cells were treated with (I) ST implant, (II) 2-NST implant, (III) 10-NST implant, (IV) 20-NST implant, (V) DOX, (VI) combination of DOX and 20-NST implant. The NST implant showed a strong anticancer effect as the concentration of loaded NaOH increased, and the combination of the 20-NST implant and DOX showed the most effective cell therapy (Fig. 3B). This result confirms that NaOH not only induces necrosis but also that alkalization by NaOH can increase the therapeutic effect of DOX [31].

3.3. *In vitro cell pH*

The change in pH due to NST implant was confirmed by measuring the pH of the cell medium. Cells were treated with DOX and NST implant at different concentrations (2-NST, 10-NST and 20-NST implants), and pH was measured over time. After 1 h of treatment, the pH value of the 2-NST implant was 7.86, that of the 10-NST implant was 8.13, and that of 20-NST was 8.5. Alkalization by the NST implant was also confirmed through a color change in the DMEM (Fig. 4A–4C) The higher the concentration of NaOH in the NST implant, the stronger the alkalinization of the cell medium. DOX caused a change in pH that was not different from that of the control, indicating that DOX did not significantly affect the pH of the medium. Because the cell culture medium contains buffer substances such as various proteins and amino acids that show a pH buffering effect, the change in pH was smaller than expected, even with a high-concentration NaOH treatment [34].

In addition, the alkalized culture medium tends to neutralize over time. This is because the cell medium can be neutralized with CO_2 via a bicarbonate buffer system. Because the bicarbonate buffer system of the human body can regulate the pH very quickly through respiration, the blood pH adjusted by NST implant can be expected to quickly return to the normal range and show low toxicity.

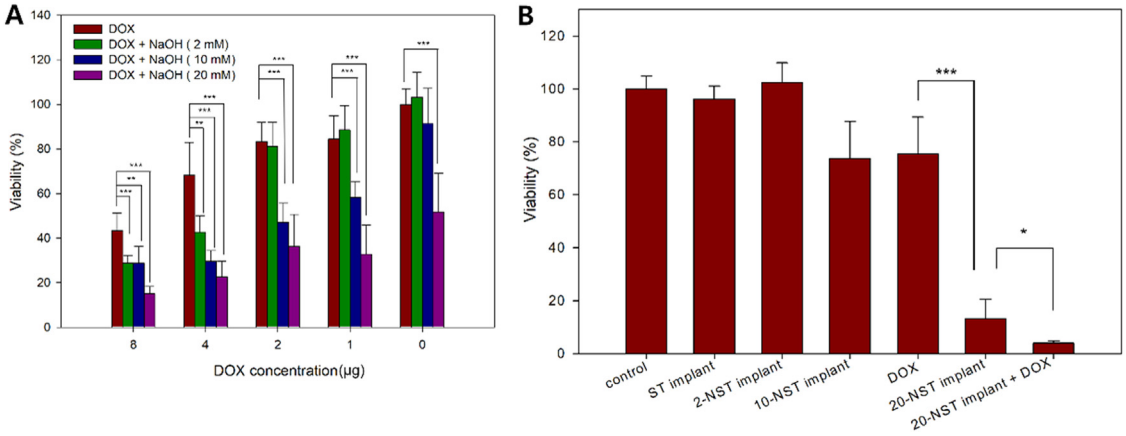


Fig. 3 – *In vitro* cytotoxicity of (A) synergistic effect of NaOH and DOX at different concentrations ($n = 8$) and (B) ST implant, 2-NST implant, 10-NST implant, 20-NST implant, DOX, and the combination of 20-NST implant and DOX ($n = 6$, ***$P < 0.001$ **$P < 0.01$ *$P < 0.05$).

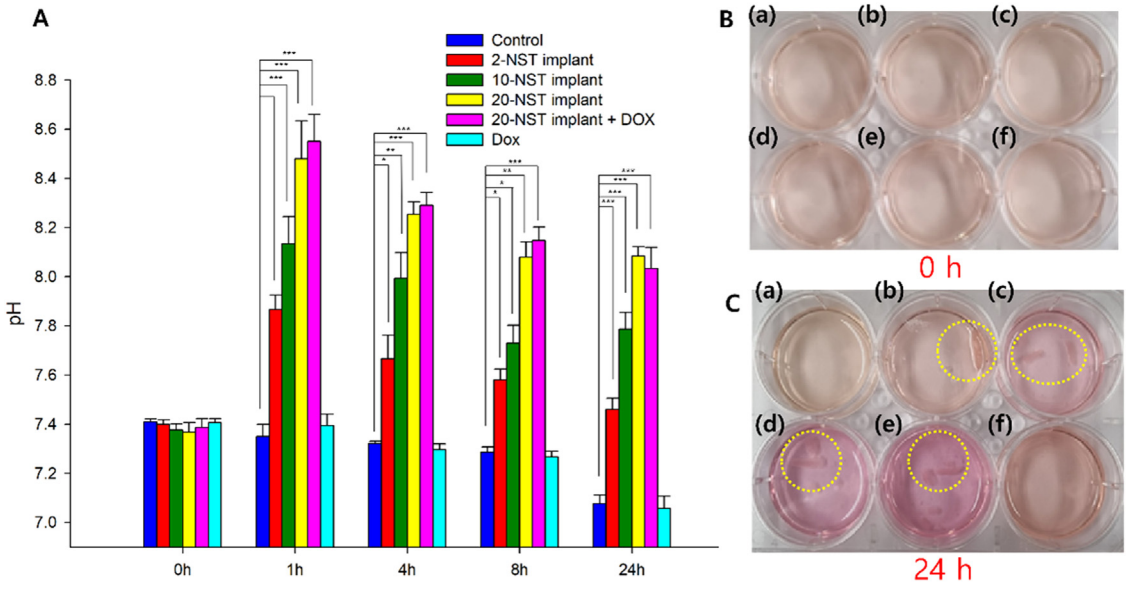


Fig. 4 – (A) pH changes of PBS (control), 2-NST implant, 10-NST implant, 20-NST implant, combination of 20-NST implant and DOX, and DOX (n = 3). (B) Monitoring of color change of DMEM media of (a) PBS, (b) 2-NST implant, (c) 10-NST implant, (d) 20-NST implant, (e) combination of 20-NST implant and DOX, and (f) DOX at 0 h and 24 h. The yellow circle indicates the floating NST implant that absorbed the medium, and therefore, swelled.

3.4. *The cellular uptake of DOX at different pH levels*

The uptake of DOX into cells varies with pH. An acidic environment can inhibit the cellular uptake of DOX through ionization. To validate this hypothesis, we examined the intracellular uptake of DOX at different pH levels. The strongest fluorescence signal indicating the maximum cellular DOX uptake was detected at pH 8.0, and the fluorescence dropped as the pH decreased (Fig. 5). These results suggest the reduction in intracellular uptake of DOX in the acidic environment, which can be increased through alkalinization

3.5. *In vivo toxicity and tumor necrosis of 20-NST implant and NaOH solution*

The toxicity of the 20-NST implant and NaOH solutions was evaluated by subcutaneous injection in mice. The NaOH solution was rapidly absorbed throughout the body and caused toxicity, resulting in death of the mice within 1 h. In contrast, the 20-NST implant caused a wound only in the surrounding tissue, which healed within 6 weeks (Fig. 6A). The slower release of NaOH from the 20-NST implant allowed an acid-base reaction to occur with the surrounding tissue, which neutralized the released NaOH and prevented systemic toxicity.

Unlike normal tissues, tumors possess a dense extracellular matrix (ECM) and abnormal blood vessels. As a result, drugs administered intratumorally tend to remain within the tumor for extended periods. Consequently, when the 20-NST implant was injected intratumorally, it was observed that the majority of the necrosis was localized to the tumor, sparing the normal tissue (Fig. 6B).

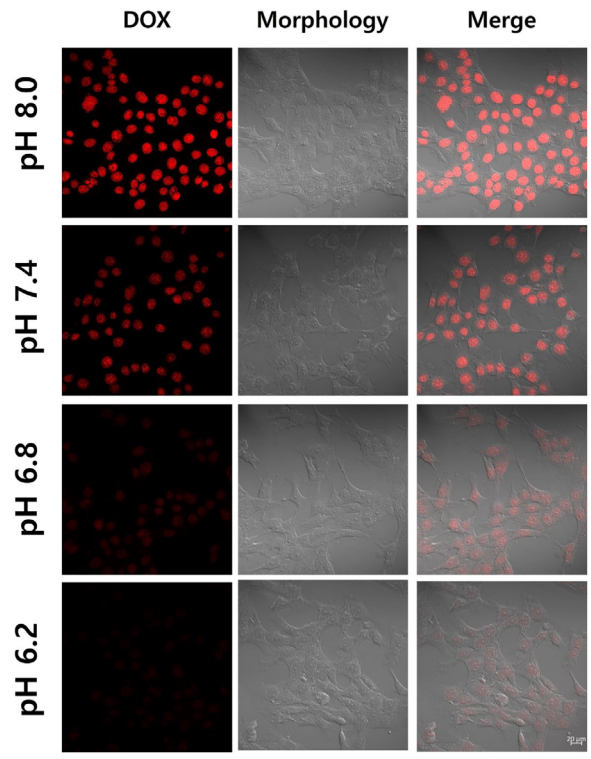


Fig. 5 – The cellular uptake of DOX at different pH (pH 8.0, 7.4, 6.8, and 6.2).

These results suggest that 20-NST implants may effectively deliver NaOH to tumors and exert anticancer effects without causing systemic toxicity. Starch can also be degraded *in*

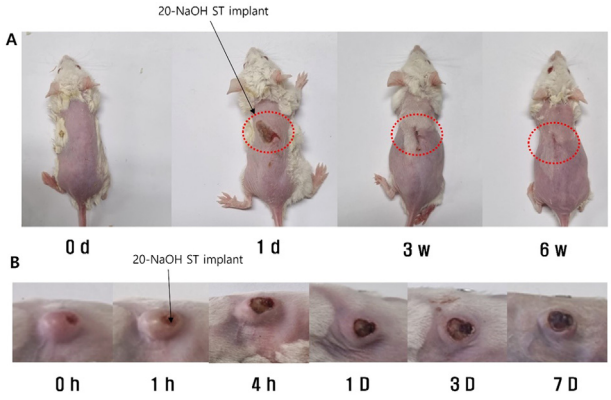


Fig. 6 – Photograph showing the (A) *in vivo* toxicity and (B) necrosis in the tumor after 20-NST implantation in mice.

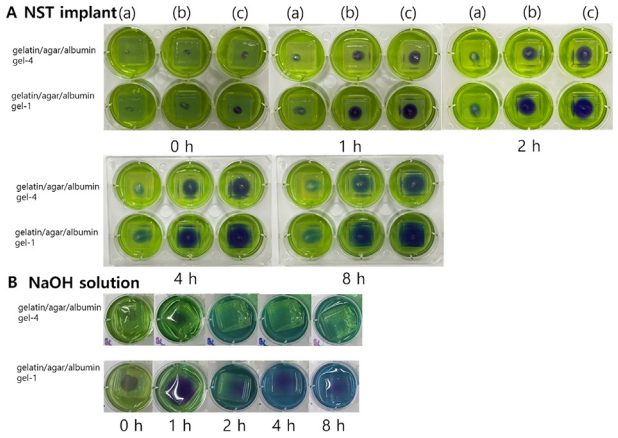


Fig. 7 – NaOH distribution of (A) (a) 2-NST implant, (b) 10-NST implant, and (c) 20-NST implant and (B) NaOH solution injected into gelatin/agar/albumin gel-1 and gelatin/agar/albumin gel-4 at different time.

vivo by enzymes such as amylases, glucoamylases and glucosidases. In a previous study, we confirmed that starch implants degrade in the body within a week [15].

3.6. Distribution of NaOH in gelatin/agar/albumin gel injected with NST implant

Tumors are composed of a densely structured TME [35–37]. Therefore, it is expected that when injected into the tumor, NaOH released from the NST implant will react with the TME around the NST-implant rather than being absorbed systemically. To confirm this, a gel with a dense structure was created using gelatin, agar, and (I) 2-NST, (II) 10-NST, (III) 20-NST implants, and (IV) 3 µl NaOH solution were injected [38–40]. A universal indicator was used to monitor pH changes (Neutral: green color; Weak alkali: blue color; Strong alkali: violet color). The results showed that the pH change only occurred slowly around (I) 2-NST, (II) 10-NST, (III) 20-NST implants and were limited to the gel (tumor mimic), with no pH change observed in the external solution (blood mimic) over a period of 8 h. Additionally, it was confirmed that higher concentrations of NaOH cause greater changes in the local pH (20-NST > 10-NST > 2-NST implant). Moreover, the change in pH was less in gelatin/agar/albumin gel-4 than in gelatin/agar/albumin gel-1 (Fig. 7). As the albumin concentration is lower in gelatin/agar/albumin gel-1 (10 mg/ml) than that in gelatin/agar/albumin gel-4 (40 mg/mL), this result may be attributable to the buffering action of albumin. Because other substances that can cause various buffering actions, such as albumin, exist in the body, it can be concluded that the effect of the released NaOH can be neutralized *in vivo*. Overall, based on the pH changes observed in the gel, it is expected that the implantation of the 20-NST implant into a tumor will only result in local alkalinization of the surrounding tumor without causing any systemic toxicity. However, the NaOH solution diffused from the gel into the external solution, which suggests that administration of the NaOH solution can potentially induce systemic toxicity owing to its tendency to spread rapidly. In addition, greater alteration in the pH was detected in gelatin/agar/albumin gel-1, containing less albumin, than in gelatin/agar/albumin gel-4 with a high albumin concentration; it indicates that proteins in the body neutralize NaOH.

3.7. 20-NST implant-induced variation in pH of blood and tumor

Before administration of the 20-NST implant, the pH values recorded in the tumor core, tumor periphery, and normal tissue 2 cm away from the tumor were 6.85, 6.87 and 7.38, respectively (Fig. 8). A mild acidic environment was detected in both the tumor core and its periphery. The pH of the tumor core increased significantly (pH >10) up to the alkaline range immediately after injecting the 20-NST implant; however, it subsequently decreased over time. The pH of the peripheral tumor tissue exhibited slight alkalinization but subsequently decreased over time. Contrastingly, the pH of the blood and normal tissue remained neutral, regardless of the 20-NST implant injection. These results demonstrate that the 20-NST implant can induce necrosis through high alkalinization around the site of injection (tumor core) and enhance DOX activity through alkalization of the acidic peripheral tumor tissue. As DOX exhibits greater intracellular uptake at an alkaline pH than in an acidic environment, 20-NST implant-induced alkalization of the tumor periphery enhances cellular DOX absorption. This phenomenon can enhance the therapeutic effect in the peripheral areas of the tumor, not directly affected by the necrotic action of NaOH from the implant. Additionally, the stable pH of the blood and normal tissue suggests that the NaOH released from the 20-NST implant was not absorbed into the body and neutralized within the tumor. These outcomes suggest that the 20-NST implant can effectively deliver NaOH to the tumor, yielding therapeutic benefits, with reduced risk of systemic side effects.

3.8. In vivo tumor therapy

To assess the anticancer effects, 4T1 tumor-bearing mice were used. When the tumors reached a size of 200–300 mm^3, the

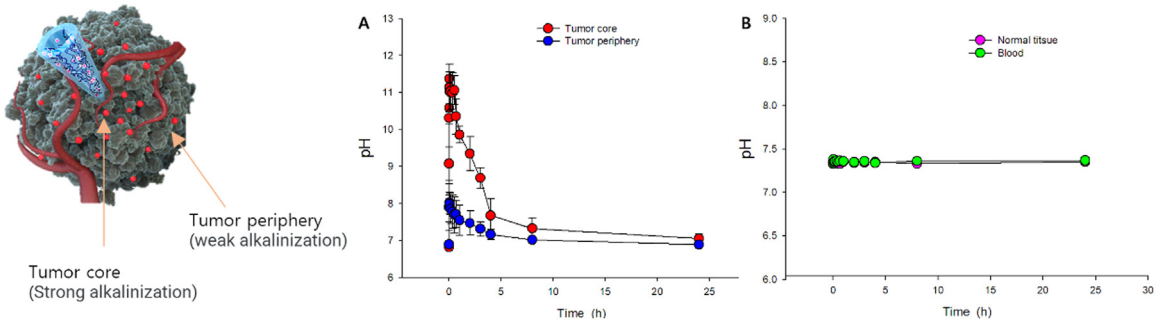


Fig. 8 – (A) Scheme of tumor core and tumor pheriphery, (B) pH change in the tumor core and tumor periphery, (C) normal tissue and blood.

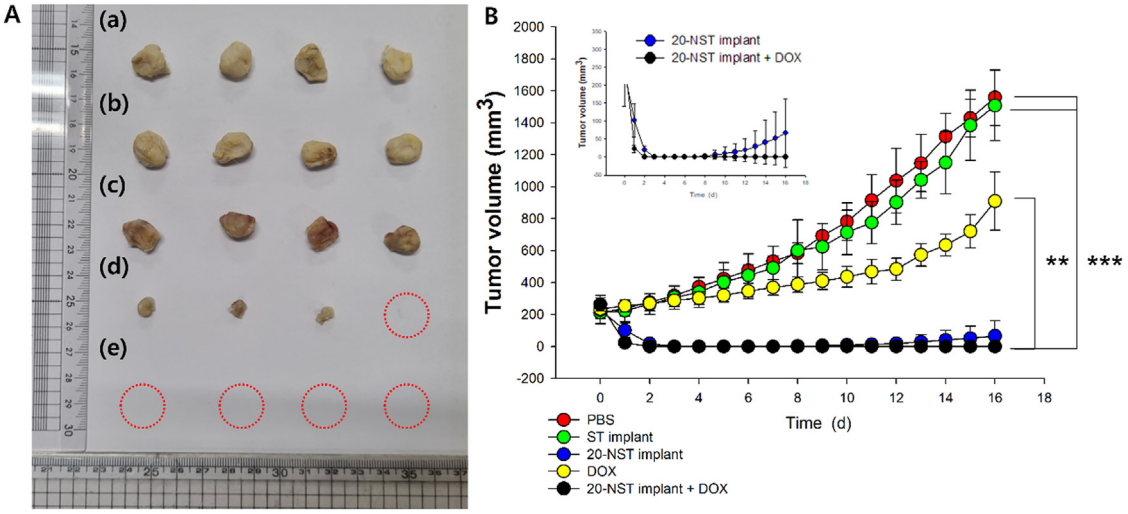


Fig. 9 – (A) Photographs of tumors excised from (a) PBS, (b) ST implant, (c) DOX, (d) 20-NST implant, and (e) combination of 20-NST implant and DOX. (B) Tumor volumes in 4T1-bearing mice with different treatment groups ($n = 4$, ***$P < 0.001$ ** $P < 0.01$).

mice were treated with (I) PBS, (II) ST-implant (III) 20-NST implants, (IV) DOX, (V) combination of 20-NST implants and DOX. The results showed that the ST implant-treated group had a tumor size of 1507.68 ± 219.35 mm^3, which was similar to that of the PBS-treated group (1558.49 ± 174.41 mm^3), indicating that the ST implant itself was not toxic. The group treated with DOX showed slight growth inhibition, with a tumor size of 909.10 ± 182.63 mm^3. In contrast, the 20-NST implant-treated group had a tumor size of 66.87 ± 94.3004 mm^3, demonstrating a significant treatment effect (Fig. 9). When combined with DOX, the 20-NST implant completely eliminated tumors. These findings suggest that 20-NST implants can effectively induce tumor necrosis through alkalization. Furthermore, the combination of 20-NST implant and DOX can kill residual tumors through a synergistic effect with 20-NST implant and DOX

3.9. *H&E staining*

The toxicity of PBS, 20-NST implant, and the combination of 20-NST implants and DOX was evaluated using H&E staining ($n = 3$). ICR mice were divided into three groups: control, 20-NST implant, and combination of 20-NST implant and DOX, each consisting of $n = 3$. After H&E staining, no toxicity was demonstrated in the liver, kidneys, lungs or heart treated with NaOH and the 20-NST implant. However, spleens treated with NaOH showed slight extramedullary hematopoiesis. Additionally, treatment with a combination of 20-NST implant and DOX moderately showed extramedullary hematopoiesis, and slightly increased megakaryocytes and red pulp (Fig. 10). This reaction is believed to result from tissue damage due to NaOH released from 20-NST implant and DOX treatment. To manage the toxicity of starch needles with added NaOH, follow-up studies that include NaOH and DOX dose-adjustment tests, as well as long-term toxicity and recovery tests, are considered necessary.

3.10. *Hematology and biochemical assay*

To assess the toxicity and immunogenisity of the combination of 20-NST implant and DOX, we evaluated hematology, biochemical assays. We used ICR mice because toxicity is commonly assessed in this species. As shown in Table 1, we obtained values from the hematology and biochemical

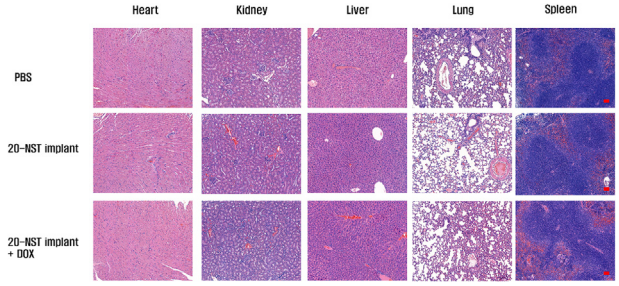


Fig. 10 – H&E staining of major organs with different treatment groups (scale bar: 50 μm).

Table 1 – Results of hematological and biochemical analyses 2 weeks after treatment of mice using PBS or a combination of 20-NST implant and DOX (n = 4, $P > 0.05$ for all markers).

Markers	PBS (control)	Combination of 20-NST implant and DOX
	Hematology	
BC(103 cells/μl)	2.3 ± 1.2	2.8 ± 1.5
RBC(106 cells/ μl)	5.0 ± 0.9	4.6 ± 0.6
HGB(g/dl)	8.3 ± 1.6	7.5 ± 0.9
HCT(%)	27.9 ± 4.8	24.8 ± 2.8
MCV(fL)	55.8 ± 1.2	54.1 ± 1.0
MCH(pg)	16.6 ± 0.5	16.3 ± 0.4
PLT(103 cells/ μl)	1158.8 ± 84.4	1169.7 ± 197.9
MCHC(g/dl)	29.8 ± 0.5	30.1 ± 0.2
	Biochemical	
TP (g/dl)	3.9 ± 0.4	4.1 ± 0.1
ALB (g/dl)	2.5 ± 0.2	2.6 ± 0.1
BUN (mg/dl)	16.6 ± 1.2	14.5 ± 2.3
CRE (mg/dl)	0.26 ± 0.03	0.25 ± 0.03
TBIL (mg/dl)	0.063 ± 0.013	0.060 ± 0.048
AST (U/l)	120.1 ± 18.5	110.9 ± 31.5
ALT (U/l)	106.0 ± 52.3	94.0 ± 22.7

assays. The data showed no statistically significant difference between the NST implant-treated mice and the control group. These results suggest that the combination of 20-NST implant and DOX does not induce systemic toxicity or immune response at hematology and biochemical assay.

3.11. *TUNEL assay of tumors*

H&E staining and the TUNEL assay was used to confirm cell death in tumor cells. The groups treated with 20-NST implants and the combination of 20-NST implants and DOX showed significantly more TUNEL-positive cells than the groups treated with PBS, ST implants, and DOX. Similarly, H&E staining results showed stronger necrosis in the 20-NST implant and the combination of 20-NST and DOX than in the other treatment groups (Fig. 11).

These results indicated that 20-NST implants and the combination of 20-NST implants and DOX have significant anticancer effects on tumors.

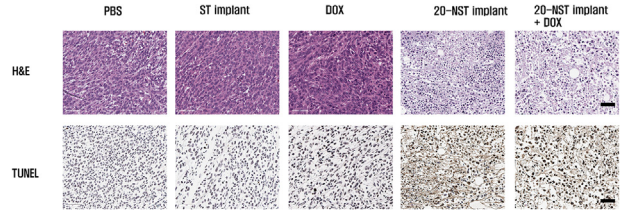


Fig. 11 – H&E staining and TUNEL assay images of tumors with different treatment groups (scale bar: 60 μm).

4. Conclusion

In summary, we developed an NaOH-loaded starch implant (NST implant). The NST implant demonstrated a delayed release of NaOH, which allowed for localized alkalization of the TME and induced necrosis, resulting in the improved cytotoxicity of DOX. In addition, the NST implant did not cause any specific toxicity to the organs, whereas the rapid uptake of the NaOH solution caused systemic toxicity, killing the mice within 1 h. The slow release of NaOH from the NST implant resulted in neutralization through an acid-base reaction with the surrounding tissue, thereby preventing systemic toxicity. These findings suggest that NST implant have great potential as a novel method for delivering NaOH to tumors and improving the therapeutic efficacy of chemotherapy.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper; The authors declare that they have no competing interests.

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References

[1] Hao GY, Xu ZP, Li L. Manipulating extracellular tumour pH: an effective target for cancer therapy. Rsc Adv 2018;8(39):22182–92.
[2] Li ZL, He P, Luo GH, Shi XC, Yuan G, Zhang B. Increased tumoral microenvironmental pH improves cytotoxic effect of pharmacologic ascorbic acid in castration-resistant prostate cancer cells. Front Pharmacol 2020;23(11):570939.
[3] De la Cruz-Lopez KG, Castro-Munoz LJ, DO Reyes-Hernandez, Garcia-Carranca A, Manzo-Merino J. Lactate in the regulation of tumor microenvironment and therapeutic approaches. Front Oncol 2019;9:01143.

[4] Sun HY, Chen L, Cao S, Liang YC, Xu Y. Warburg effects in cancer and normal proliferating cells: two tales of the same name. Genom Proteom Bioinf 2019;17(3):273–86.
[5] Lee S, Shanti A. Effect of exogenous pH on cell growth of breast cancer cells. Int J Mol Sci 2021;22(18):9910.
[6] Zhong X, He X, Wang Y, Hu Z, Huang H, Zhao S, et al. Warburg effect in colorectal cancer: the emerging roles in tumor microenvironment and therapeutic implications. J Hematol Oncol 2022;15(1):160.
[7] Robey IF, Baggett BK, Kirkpatrick ND, Roe DJ, Dosescu J, Sloane BF, et al. Bicarbonate increases tumor pH and inhibits spontaneous metastases. Cancer Res 2009;69(6):2260–8.
[8] Ando H, Ikeda A, Tagami M, Matsuo NCA, Shimizu T, Ishima Y, et al. Oral administration of sodium bicarbonate can enhance the therapeutic outcome of Doxil(R) via neutralizing the acidic tumor microenvironment. J Control Release 2022;350:414–20.
[9] Abumanhal-Masarweh H, Koren L, Zinger A, Yaari Z, Krinsky N, Kaneti G, et al. Sodium bicarbonate nanoparticles modulate the tumor pH and enhance the cellular uptake of doxorubicin. J Control Release 2019;296:1–13.
[10] O'Brien C, Ignaszak A. Advances in the electrochemical treatment of cancers and tumors: exploring the current trends, advancements, and mechanisms of electrolytic tumor ablation. Chemelectrochem 2020;7(19):3895–904.
[11] Perkons NR, Stein EJ, Nwaezeapu C, Wildenberg JC, Saleh K, Itkin-Ofer R, et al. Electrolytic ablation enables cancer cell targeting through pH modulation. Commun Biol 2018;17(1):48.
[12] Kim A, Lee SK, Parupudi T, Rahimi R, Song SH, Park MC, et al. An ultrasonically powered implantable microprobe for electrolytic ablation. Sci Rep 2020;10(1):1510.
[13] Emoto Y, Yoshizawa K, Shikata N, Tsubura A, Nagasaki Y. Autopsy results of a case of ingestion of sodium hydroxide solution. J Toxicol Pathol 2016;29(1):45–7.
[14] Von Euler H, Soerstedt A, Thorne A, Olsson JM, Yongqing G. Cellular toxicity induced by different pH levels on the R3230AC rat mammary tumour cell line. An in vitro model for investigation of the tumour destructive properties of electrochemical treatment of tumours. Bioelectrochemistry 2002;58(2):163–70.
[15] Lee CK. Development of injectable and biodegradable needle-type starch implant for effective intratumoral drug delivery and distribution. Int J Nanomedicine 2022;17:4307–19.
[16] Park S, Kim J, Lee CK. Injectable rapidly dissolving needle-type gelatin implant capable of delivering high concentrations of H_2O_2 through intratumoral injection. Biomed Pharmacother 2022;156:113910.
[17] Lemos PVF, Marcelino HR, Cardoso LG, de Souza CO, Druzian JI. Starch chemical modifications applied to drug delivery systems: from fundamentals to FDA-approved raw materials. Int J Biol Macromol 2021;184:218–34.
[18] Jin N, Kong DD, Wang HY. Effects of temperature and time on gelatinization of corn starch employing gradient isothermal heating program of rapid visco analyzer. J Food Process Eng 2019;42(7):e13264.
[19] Hu XT, Xu XM, Jin ZY, Tian YQ, Bai YX, Xie ZJ. Retrogradation properties of rice starch gelatinized by heat and high hydrostatic pressure (HHP). J Food Eng 2011;106(3):262–6.
[20] Waghule T, Singhvi G, Dubey SK, Pandey MM, Gupta G, Singh M, et al. Microneedles: a smart approach and increasing potential for transdermal drug delivery system. Biomed Pharmacother 2019;109:1249–58.
[21] Chi CD, He YJ, Jiao WJ, Wang HW, Tan XY. Hierarchical structural transformation of corn starch in NaOH solution at room temperature. Ind Crop Prod 2022(178):114672.
[22] Kuleasan S, Tekin A. Alkaline neutralization of crude soybean oil by various adsorbents. Eur J Lipid Sci Tech 2008;110(3):261–5.
[23] Ling BP, Chen HT, Liang DY, Lin W, Qi XY, Liu HP, et al. Acidic pH and high-H_2O_2 dual tumor microenvironment-responsive nanocatalytic graphene oxide for cancer selective therapy and recognition. ACS Appl Mater Interfaces 2019;11(12):11157–66.
[24] Boedtkjer E, Pedersen SF. The acidic tumor microenvironment as a driver of cancer. Annu Rev Physiol 2020;82:103–26.
[25] Krieg BJ, Taghavi SM, Amidon GL, Amidon GE. *In vivo* predictive dissolution: transport analysis of the CO_2, bicarbonate *in vivo* buffer system. J Pharm Sci Us 2014;103(11):3473–90.
[26] Martin NK, Gaffney EA, Gatenby RA, Gillies RJ, Robey IF, Maini PK. A mathematical model of tumour and blood pHe regulation: the HCO_3-/CO_2 buffering system. Math Biosci 2011;230(1):1–11.
[27] Garcia AJ, Ramirez JM. Chemoreception: keeping carbon dioxide in check. Elife 2017;6:e27653.
[28] Watson PD. Modeling the effects of proteins on pH in plasma. J Appl Physiol 1999;86(4):1421–7.
[29] Krbec M, Koudelkova K, Duska F. On acid-base effects of albumin. Curr Med Res Opin 2021;37(9):1515–16.
[30] Trebinska-Stryjewska A, Swiech O, Opuchlik LJ, Grzybowska EA, Bilewicz R. Impact of medium pH on DOX toxicity toward HELA and a498 cell lines. ACS Omega 2020;5(14):7979–86.
[31] Swietach P, Hulikova A, Patiar S, Vaughan-Jones RD, Harris AL. Importance of intracellular ph in determining the uptake and efficacy of the weakly basic chemotherapeutic drug, doxorubicin. PLoS ONE 2012;7(4):e35949.
[32] Abumanhal-Masarweh H, Koren L, Zinger A, Yaari Z, Krinsky N, Kaneti G, et al. Sodium bicarbonate nanoparticles modulate the tumor pH and enhance the cellular uptake of doxorubicin. J Controlled Release 2019;296:1–13.
[33] Phuong PTT, Lee S, Lee C, Seo B, Park S, Oh KT, et al. Beta-carotene-bound albumin nanoparticles modified with chlorin e6 for breast tumor ablation based on photodynamic therapy. Colloid Surface B 2018;171:123–33.
[34] Michl J, Park KC, Swietach P. Evidence-based guidelines for controlling pH in mammalian live-cell culture systems. Commun Biol 2019;26(2):144.
[35] Katz RR, West JL. Reductionist three-dimensional tumor microenvironment models in synthetic hydrogels. Cancers (Basel) 2022;14(5):1225.
[36] Xu X, Farach-Carson MC, Jia XQ. Three-dimensional in vitro tumor models for cancer research and drug evaluation. Biotechnol Adv 2014;32(7):1256–68.
[37] Baghba R, Roshangar L, Jahanban-Esfahlan R, Seidi K, Ebrahimi-Kalan A, Jaymand M, et al. Tumor microenvironment complexity and therapeutic implications at a glance. Cell Commun Signal 2020;18(1):59.
[38] Gupta D, Santoso JW, McCain ML. Characterization of gelatin hydrogels cross-linked with microbial transglutaminase as engineered skeletal muscle substrates. Bioengineering Basel 2021;8(1):6.
[39] Wakhet S, Singh VK, Sahoo S, Sagiri SS, Kulanthaivel S, Bhattacharya MK, et al. Characterization of gelatin-agar based phase separated hydrogel, EmulGel and Bigel: a comparative study. J Mater Sci Mater M 2015;26(2):118.
[40] Yang G, Xiao ZH, Ren XM, Long HY, Qian H, Ma KL, et al. Enzymatically crosslinked gelatin hydrogel promotes the proliferation of adipose tissue-derived stromal cells. PeerJ 2016;27(4):2497.