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Kai Hu

The First Affiliated Hospital of University of Science and Technology of China

Guohong Wu

The First Affiliated Hospital of University of Science and Technology of China

Wei Wen

The First Affiliated Hospital of University of Science and Technology of China

Can Zhang

zhangcan84@163.com

The First Affiliated Hospital of University of Science and Technology of China

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Enhanced Lesion Depth and Preserved Deep Thermal Safety of Sequential Radiofrequency and Pulsed Field Ablation: An Ex Vivo Porcine Heart Study

Kai Hu¹, Guohong Wu¹, Wei Wen¹, Can Zhang^{1*}

¹ Department of Cardiology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, China

*Correspondence: Can Zhang, zhangcan84@163.com

Abstract

Background Catheter ablation has become a standard rhythm-control strategy for atrial fibrillation, but creating lesions deep enough to be effective—without risking thermal damage to nearby structures, especially the esophagus and left atrial posterior wall—remains a difficult clinical problem. Radiofrequency ablation (RFA) reliably produces thermal coagulative necrosis, yet the same thermal mechanism puts periesophageal tissue at risk for deep injury. Pulsed field ablation (PFA) works by irreversible electroporation and creates myocardial-selective, non-thermal injury, though the lesions it produces tend to be relatively shallow. Sequential dual-energy ablation, in which RFA and PFA are delivered in the same procedure, could combine the greater depth of thermal coagulation with the tissue selectivity of electroporation. How the order of energy delivery affects lesion formation, deep tissue temperature dynamics, and the cell death mechanisms involved, however, has not been systematically examined.

Methods We used an ex vivo porcine heart model to compare lesion formation and deep thermal safety across four ablation strategies: RFA alone (ablation index [AI] 350, 400, 450, and 500), PFA alone (1 or 2 discharges), RFA followed by PFA (RFA+PFA), and PFA followed by RFA (PFA+RFA), with six repetitions per parameter combination ($n = 6$). Lesion depth and width were measured with triphenyltetrazolium chloride (TTC) staining. Tissue injury patterns and cell death modes were assessed by hematoxylin-eosin (HE) staining and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. Deep tissue temperature was recorded continuously at 3 mm and 7 mm below the ablation site using PT100 platinum resistance temperature probes (1.5 mm diameter, 2 cm length, accuracy $\pm 0.1^{\circ}\text{C}$, 0.1 s sampling interval); peak temperature (T_{max}), temperature rise (dT), maximum heating rate (dT/dt), and the 7 mm-to-3 mm attenuation ratio were calculated as thermal safety indicators.

Results In the RFA-only group, lesion size grew as AI increased, and AI 500 produced the deepest and widest lesions. PFA-only lesions were comparatively small but showed the typical injury pattern of electroporation. Sequential dual-energy ablation caused more myocardial damage than either energy alone; the RFA+PFA group (RFA AI 500 + PFA 2 discharges) reached the greatest lesion depth (approximately 6.9 mm), which was significantly deeper than RFA AI 500 alone. Temperature recordings showed a 4–11 $^{\circ}\text{C}$ rise at 3 mm across groups, whereas at 7 mm the rise was much smaller ($dT < 1^{\circ}\text{C}$ in all groups), with 7 mm-to-3 mm attenuation ratios well below 1. The maximum heating rate at 3 mm was highest with RFA (approximately 17.90 $^{\circ}\text{C/s}$) and lowest with PFA (approximately 8.58 $^{\circ}\text{C/s}$). On histology, RFA mainly produced thermal coagulative necrosis with minimal TUNEL positivity (around 2%), whereas PFA led to apoptosis-like changes and the highest proportion of TUNEL-positive cells. The RFA+PFA group showed primarily necrotic injury, while the PFA+RFA group had a mixture of necrosis and apoptosis-like changes.

Conclusion Sequential RFA and PFA produces larger ex vivo myocardial lesions than either modality alone, mainly by increasing depth, while heat spread to 7 mm remains limited—pointing to an acceptable thermal safety margin for nearby structures. The order of energy delivery matters: it shapes lesion morphology, temperature response, and cell death patterns, with RFA+PFA producing mainly necrosis and PFA+RFA retaining some apoptosis-like features. These preclinical data support further development of dual-energy ablation and suggest that optimizing the energy sequence is key to balancing efficacy and safety.

Keywords: radiofrequency ablation; pulsed-field ablation; thermal safety; dual-energy ablation; lesion depth; ex vivo model

*Correspondence: [Corresponding Author Email]

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Background

Catheter ablation has become an important rhythm-control strategy for patients with atrial fibrillation who respond poorly to pharmacological therapy. Radiofrequency ablation (RFA) can produce relatively well-defined coagulative myocardial necrosis lesions through thermal effects. However, because the posterior wall of the left atrium is adjacent to the esophagus, balancing lesion depth with the thermal safety of neighboring tissues is particularly critical. Although high-power radiofrequency ablation can improve ablation efficiency and increase the likelihood of transmural injury, thermal conduction may still affect structures such as the esophagus and phrenic nerve. Atrioesophageal fistula has a low incidence but a high mortality rate; therefore, deep tissue temperature changes and histological injury characteristics should be monitored simultaneously [1, 2].

Pulsed field ablation (PFA) is mainly based on irreversible electroporation and is characterized by myocardial tissue selectivity and nonthermal injury. Existing studies have shown that PFA has favorable procedural efficiency and short-term safety in atrial fibrillation ablation, with efficacy in some studies approaching that of radiofrequency or cryoballoon ablation [3-6]. PFA applied to posterior left atrial wall isolation can achieve a high acute isolation rate, but lesion continuity, lesion depth, long-term efficacy, and effects on left atrial function still require further evaluation [7, 8]. The lesion morphologies produced by different energy modalities are not completely consistent, suggesting that their efficacy advantages and safety limitations may differ [9].

Sequential dual-energy ablation using radiofrequency and pulsed field energy has potential research value. RFA can produce stable thermally coagulated lesions, while PFA may supplement the range of cellular injury. The key question is whether lesion enhancement is accompanied by increased deep tissue temperature elevation. This study used an ex vivo porcine myocardial model to compare four ablation modes: RFA alone, PFA alone, PFA followed by RFA, and RFA followed by PFA. A circular catheter was used for PFA delivery, and a linear catheter was used for RFA delivery. The evaluation framework referred to the approach used by Zito et al., in which tissue surface temperature and temperatures at depths of 3 mm and 7 mm were monitored under a perfusion condition of 30 mL/min [10, 11]. Lesion extent and cellular injury were assessed by TTC, hematoxylin and eosin (HE), and TUNEL staining. Peak temperature, temperature rise rate, and temperature recovery time were used to evaluate the risk of deep thermal conduction, providing experimental evidence for optimizing ablation parameters in high-risk regions such as the posterior wall of the left atrium.

Methods

Experimental Materials and Establishment of the Ex Vivo Porcine Heart Model

Fresh porcine hearts were obtained from a local slaughterhouse within 2 hours of animal sacrifice. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of University of Science and Technology of China, and all procedures were performed in accordance with institutional guidelines for the

care and use of laboratory animals. The main materials included ex vivo porcine hearts, normal saline, a tissue fixation device, high-response microtemperature probes, TTC staining reagents, HE staining reagents, a TUNEL detection kit, and routine pathological sampling instruments. After collection, the porcine hearts were promptly transported to the laboratory. The cardiac chambers and myocardial surfaces were thoroughly rinsed with normal saline to remove residual blood and blood clots, and the tissues were maintained in a constant-temperature and humid environment to preserve tissue condition.

Myocardial regions with relatively uniform thickness, flat surfaces, and convenient catheter contact were selected as ablation sites. Before ablation, each ablation point was positioned and marked. Adequate spacing was maintained between adjacent points to reduce lesion overlap and the influence of residual heat on measurement results. During model establishment, catheter contact was kept relatively stable. A circular catheter was used for pulsed field ablation, and a linear catheter was used for radiofrequency ablation, thereby improving the reliability of comparisons among different ablation modes [12-14]. The experimental setup and temperature probe placement are shown in Fig. 1.

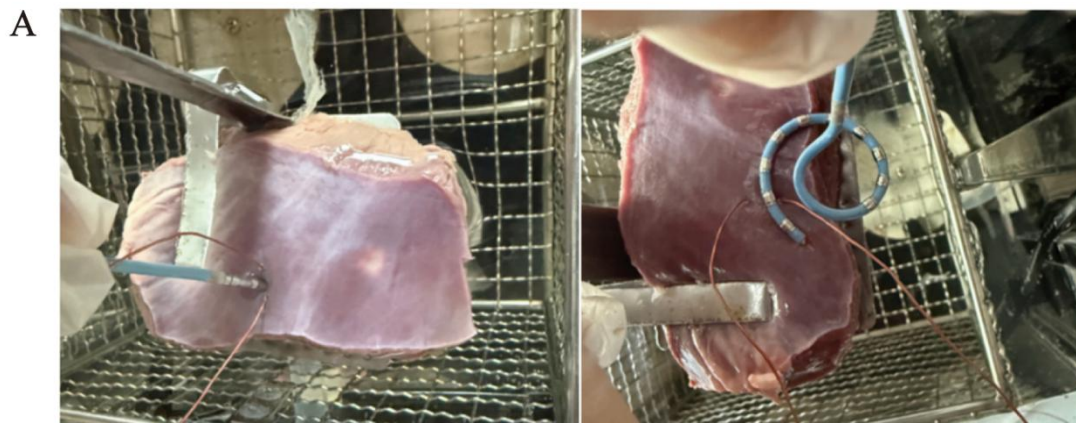


Fig. 1 Schematic diagram of the ex vivo experimental setup showing the porcine heart model, ablation catheter placement, and temperature probe positioning at 3 mm and 7 mm depths beneath the ablation site.

To evaluate deep thermal conduction, PT100 platinum resistance temperature probes (1.5 mm diameter, 2 cm length, accuracy $\pm 0.1^{\circ}\text{C}$) were implanted at depths of 3 mm and 7 mm directly beneath the ablation point. Temperature data were continuously recorded by a multi-channel temperature logger with a sampling interval of 0.1 seconds. Baseline temperature was recorded before ablation, and temperature-time curves were continuously collected during ablation. This setting was based on recent PFA temperature dynamic studies that evaluated tissue surface and deep temperature changes [15]. Each ablation point served as an independent observation unit and was treated with RFA alone, PFA alone, PFA + RFA, or RFA + PFA. Each group of parameters was repeated six times. Based on previous ex vivo ablation studies [10], a sample size of 6 per group was calculated to provide 80% power to detect a 20% difference in lesion

depth between groups at $\alpha=0.05$.

After ablation, lesion tissues were harvested according to their assigned numbers. TTC staining was used to measure lesion depth and width, while HE staining and TUNEL staining were performed to evaluate tissue morphology and the level of cell death. During PFA experiments, tissue moisture and medium stability were maintained to reduce the influence of impedance changes on electric field effects and lesion formation [16].

Ablation Devices and Energy Parameter Settings

In this study, a linear catheter was used to deliver radiofrequency energy, and a circular catheter was used to deliver pulsed field energy. Radiofrequency ablation was performed using the STSF radiofrequency system. The power was fixed at 40 W, and the radiofrequency dose was controlled by the ablation index endpoint, which was set at 350, 400, 450, and 500. Previous studies have suggested that ablation index-guided radiofrequency ablation helps control the intensity of energy delivery, and that lesion formation is closely related to power, application time, catheter stability, and contact conditions [17, 18].

Pulsed field ablation was performed using the Varipulse PFA system with discharge delivered through a circular catheter. The irrigation flow rate was fixed at 30 mL/min, and the number of discharges was set at one or two. Related studies have shown that the configuration and pulse parameters of circular or variable-loop PFA catheters can influence energy delivery and procedural workflow [19, 20]. Sequential combined ablation included two modes: PFA + RFA and RFA + PFA, allowing comparison of lesion formation and deep temperature changes under different energy sequences. Because the mode of energy delivery affects the evaluation of ablation efficiency and safety, catheter type, energy modality, and delivery sequence were controlled in this study to compare differences between single-energy and sequential dual-energy ablation [21].

Experimental Grouping

This study included four groups: the RFA-alone group, PFA-alone group, PFA + RFA group, and RFA + PFA group. In the RFA-alone group, radiofrequency energy was delivered using a linear catheter, with ablation index values set at 350, 400, 450, and 500, to observe lesion formation and deep temperature changes under different radiofrequency doses. In the PFA-alone group, pulsed field energy was delivered using a circular catheter, with one or two discharges, to evaluate lesion characteristics and temperature responses produced by PFA alone. In the PFA + RFA group, one or two PFA discharges were first delivered using the circular catheter, followed by RFA using the linear catheter at ablation index values of 350, 400, 450, or 500. In the RFA + PFA group, RFA at the corresponding ablation index value was first completed, followed by one or two PFA discharges. Each parameter combination was repeated six times. The single-energy groups were used to establish the basic controls for RFA and PFA, whereas the sequential dual-energy groups were used to compare the effects of different delivery sequences on lesion depth, lesion width, tissue injury characteristics, and deep temperature changes [22-26].

TTC Staining and Measurement of Lesion Depth and Width

After ablation, the tissue surface was immediately rinsed three times with precooled 0.1 mol/L PBS (pH 7.4) to remove residual electrode fluid and exudates from the injured area. The myocardial tissue from the ablation region was then cut into uniform slices 2–3 mm thick using sterile surgical scissors to ensure adequate penetration of the TTC staining solution. Under light-protected conditions, TTC powder was dissolved in 0.1 mol/L PBS (pH 7.4) to prepare a 1% TTC staining solution. The myocardial slices were completely immersed in the solution and incubated in a 37°C constant-temperature incubator protected from light for 20 minutes. During incubation, the slices were gently shaken every 5 minutes to reduce tissue adherence and uneven staining.

After incubation, residual staining solution on the slice surfaces was rinsed off with PBS three times, 1 minute each time. The slices were then immediately placed in 4% paraformaldehyde fixative and fixed at room temperature for 15 minutes. The fixation time did not exceed 30 minutes to avoid affecting the macroscopic observation of TTC reduction products. After staining, the slices were observed and photographed under a microscope. Bright red areas were defined as viable regions, dark red areas as injury transition zones, and white or pale-yellow areas as completely injured regions. The completely injured area and its boundary were used to determine lesion extent. The maximum lesion depth and maximum lesion width of each ablation point were measured to compare injury size among the RFA group, PFA group, RFA + PFA group, and PFA + RFA group, as well as the effects of different ablation sequences on lesion formation [27-31].

HE Staining and Histomorphological Observation

After ablation, myocardial tissues from the lesion regions of each group were collected and fixed in 10% neutral formalin for at least 1 hour. After paraffin embedding, 10 µm sections were prepared, stained sequentially with hematoxylin and eosin, and observed under a light microscope. HE staining was mainly used to evaluate myocardial cell morphological changes under different ablation methods. In the RFA group, the main observations included necrotic manifestations such as cellular swelling, irregular morphology, nuclear dissolution or disappearance, and cell membrane rupture. In the PFA group, the main observations included apoptosis-related manifestations such as cell shrinkage, nuclear pyknosis, chromatin condensation, and relatively intact cell membranes. In the RFA + PFA and PFA + RFA groups, the distribution of necrotic and apoptosis-like changes, injury boundaries, and degree of tissue structural disruption were compared to evaluate the effects of different energy sequences on myocardial tissue injury characteristics.

TUNEL Staining and Calculation of the Positive Cell Rate

After ablation, myocardial tissues from the lesion regions of each group were collected and fixed in 10% neutral formalin for at least 1 hour. After paraffin embedding, the tissues were cut into 10 µm sections, followed by sectioning, spreading, mounting, and baking. Before the TUNEL reaction, excess liquid around the sections was wiped dry. The TUNEL reaction mixture was prepared according to the reagent kit instructions. Terminal transferase and labeled dUTP were mixed and applied dropwise onto the tissue sections. The sections were then incubated at 37°C for 60 minutes in the dark. The entire procedure was protected from light to prevent quenching of the fluorescent marker.

After the reaction, the sections were observed and photographed under a fluorescence microscope. TUNEL-positive cells showed obvious fluorescent signals. Five random fields at 400× magnification were selected from each section, and the numbers of TUNEL-positive cells and total cells were counted separately. The TUNEL-positive cell rate was calculated using the following formula: TUNEL-positive cell rate = number of positive cells / total number of cells × 100%. TUNEL staining is commonly used to evaluate apoptosis or DNA fragmentation levels in myocardial tissue and, together with HE morphological observation, can reflect the degree of myocardial injury [32-36].

Statistical Analysis

Statistical analysis was performed using SPSS 26.0 (IBM, Armonk, NY) and GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA). Measurement data were expressed as mean ± standard deviation. Normality was assessed using the Shapiro-Wilk test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for pairwise comparisons. If the data did not conform to a normal distribution, nonparametric Kruskal-Wallis test was used, followed by Dunn's post hoc test. TTC lesion depth and width, TUNEL-positive cell rate, and temperature indices at depths of 3 mm and 7 mm were included in the statistical analysis. HE staining results were mainly described morphologically. All statistical tests were two-tailed, and a value of $P < 0.05$ was considered statistically significant.

Results

Dynamic Changes in Deep Tissue Temperature and Thermal Safety Evaluation

Deep temperature monitoring showed that the temperature response at 3 mm differed markedly among different ablation modes, whereas the overall temperature rise at 7 mm was small. To further clarify the effects of different energy parameters and sequential modes on deep temperature changes, this study analyzed the temperature dynamic characteristics at depths of 3 mm and 7 mm in the simple RFA group, simple PFA group, and sequential dual-energy RFA→PFA and PFA→RFA groups. Peak temperature T_{max} , temperature rise ΔT , maximum heating rate dT/dt , and the 7 mm-to-3 mm temperature rise attenuation ratio were used to evaluate deep thermal safety.

Temperature Dynamics in Simple RFA Group

In the simple RFA group, the peak temperature and temperature rise at 3 mm generally increased as the AI value rose from 350 to 500 (Fig. 2). When AI=350, the representative T_{max} at 3 mm was approximately 43.11°C, and ΔT was approximately 6.22°C. When AI=400, the T_{max} at 3 mm increased to approximately 45.22°C, and ΔT increased to approximately 8.32°C. When AI=450, the T_{max} at 3 mm further increased to approximately 46.78°C, and ΔT increased to approximately 10.09°C. When AI=500, the T_{max} at 3 mm reached approximately 49.20°C, and ΔT reached approximately 11.98°C. These results indicate that the deep temperature response of simple RFA was closely related to the increase in AI value, and high

AI values could induce a more evident temperature rise at 3 mm. In contrast, temperature changes at 7 mm were relatively flat. Under each AI value, the Tmax at 7 mm was mostly maintained between approximately 35.67°C and 37.04°C, and the temperature rise was markedly lower than that at 3 mm, suggesting obvious attenuation of radiofrequency heat conduction from shallow tissue to deeper tissue.

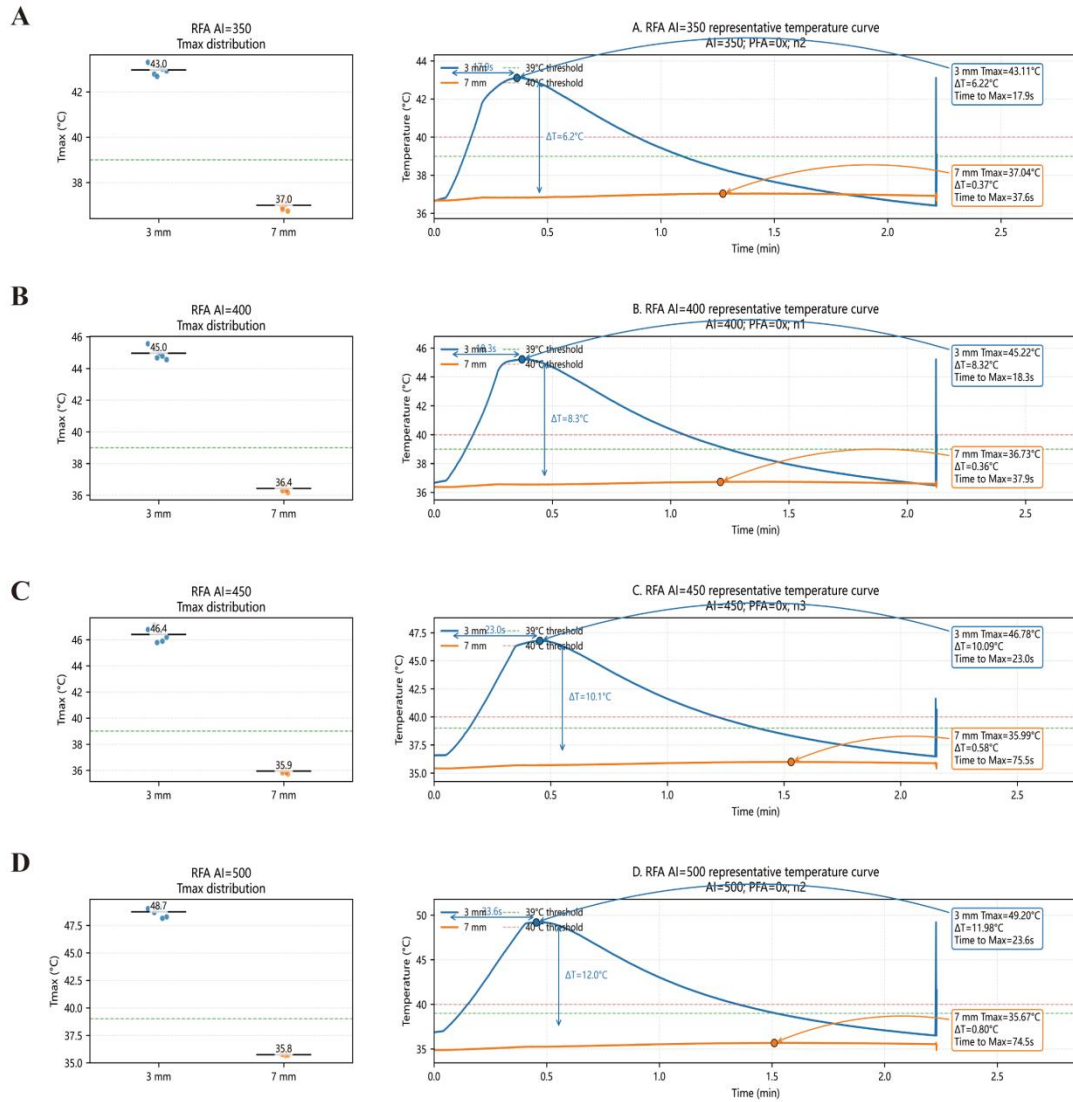


Fig. 2 Deep temperature dynamic curves at 3 mm and 7 mm under different AI values in the simple RFA group. A-D: Tmax distribution and representative temperature curves at 3 mm and 7 mm when RFA AI=350, 400, 450, and 500, respectively

Temperature Dynamics in Simple PFA Group

Both one and two PFA discharges caused measurable temperature changes at 3 mm, although the overall rise remained lower than that observed with high-AI RFA (Fig. 3). During PFA 1×, the representative Tmax at 3 mm was approximately 39.10°C, and ΔT was approximately 3.06°C. During PFA 2×, the representative Tmax at 3 mm was approximately 41.43°C, and ΔT was approximately 5.19°C. Compared with PFA 1×, PFA 2× produced a higher peak

temperature and greater temperature rise at 3 mm, indicating that increasing the number of PFA discharges could enhance the local temperature response. Temperature changes at 7 mm remained limited. The T_{max} at 7 mm was approximately 35.47°C during PFA 1× and approximately 36.94°C during PFA 2×, both of which were lower than the temperature response at 3 mm. These results suggest that, although PFA is mainly based on a nonthermal injury mechanism, multiple discharges can still produce a certain degree of local temperature change, while deep thermal conduction remains relatively small.

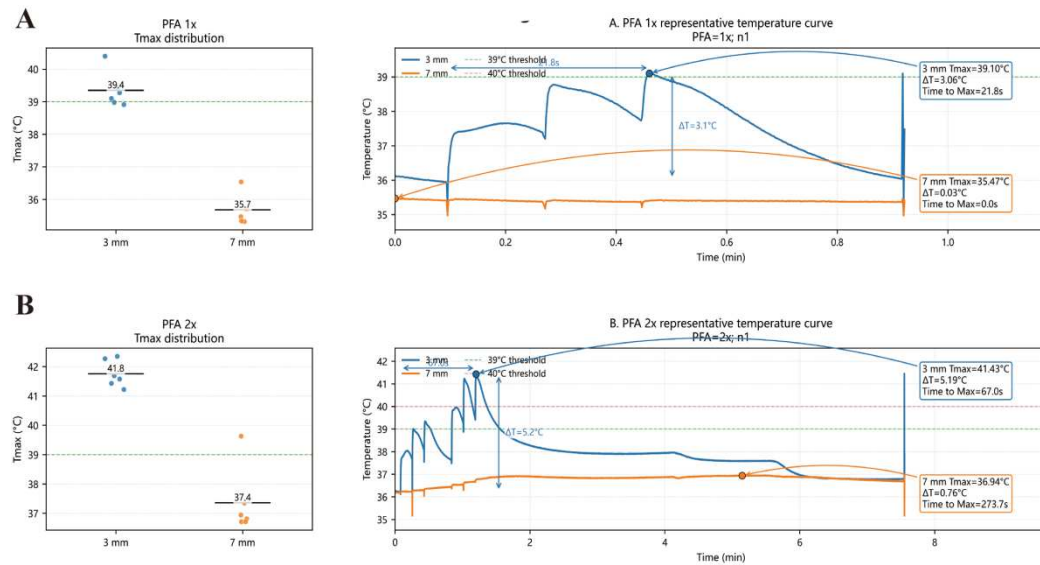


Fig.3 Deep temperature dynamic curves at 3 mm and 7 mm under different discharge numbers in the simple PFA group. A: T_{max} distribution and representative temperature curves at 3 mm and 7 mm during PFA 1×; B: T_{max} distribution and representative temperature curves at 3 mm and 7 mm during PFA 2×.

Temperature Dynamics in Sequential Dual-Energy Groups

The sequential dual-energy temperature profiles indicated a relatively evident rise at 3 mm during RFA→PFA ablation (Fig. 4, first and second rows). In the RFA→PFA group, the temperature peak at 3 mm mainly occurred during the RFA phase, followed by a certain degree of temperature fluctuation or secondary rise after PFA was added. As the AI value increased, the T_{max} at 3 mm generally increased. Under the RFA→PFA, PFA 1× condition, the T_{max} at 3 mm increased from approximately 43.0°C to 49.5°C as AI increased from 350 to 500. Under the RFA→PFA, PFA 2× condition, the T_{max} at 3 mm increased from approximately 42.9°C to 49.3°C as AI increased from 350 to 500. Temperature changes at 7 mm remained relatively flat, and the T_{max} under most conditions was approximately 35.7°C to 37.5°C, which was markedly lower than that at 3 mm.

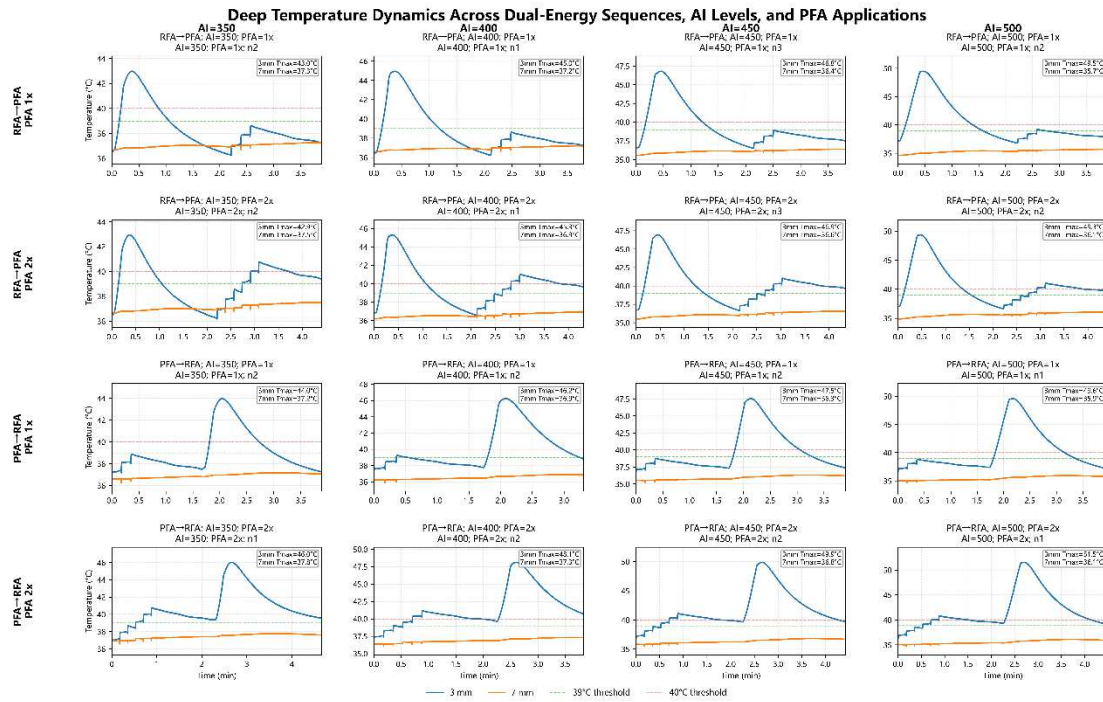


Fig. 4 Deep temperature dynamic curves at 3 mm and 7 mm under different AI values and PFA discharge combinations in sequential dual-energy ablation

First row: RFA→PFA with PFA 1× under different AI values; second row: RFA→PFA with PFA 2× under different AI values; third row: PFA→RFA with PFA 1× under different AI values; fourth row: PFA→RFA with PFA 2× under different AI values.

In the PFA→RFA configurations shown in the third and fourth rows of Fig. 4, the temperature curves usually showed a mild stepwise increase during the PFA phase, followed by the main temperature peak during the RFA phase. The most evident temperature rise at 3 mm was observed under higher AI values and PFA 2× conditions. In particular, under the PFA 2×+RFA AI=500 condition, the Tmax at 3 mm reached approximately 51.5°C, higher than most other combinations, suggesting that when PFA was performed before RFA, high-AI radiofrequency energy might further enhance the temperature response at 3 mm on the basis of the preceding PFA effect. Compared with 3 mm, the Tmax at 7 mm under all combinations remained at a relatively low level, mostly concentrated between approximately 35.9°C and 37.8°C. This indicates that, although sequential dual-energy ablation can enhance the shallow-to-middle tissue temperature response, its thermal effect conducted to the 7 mm depth remains clearly limited. The intergroup comparisons of peak temperature, temperature rise, and maximum heating rate at 3 mm and 7 mm are summarized in Fig. 5.

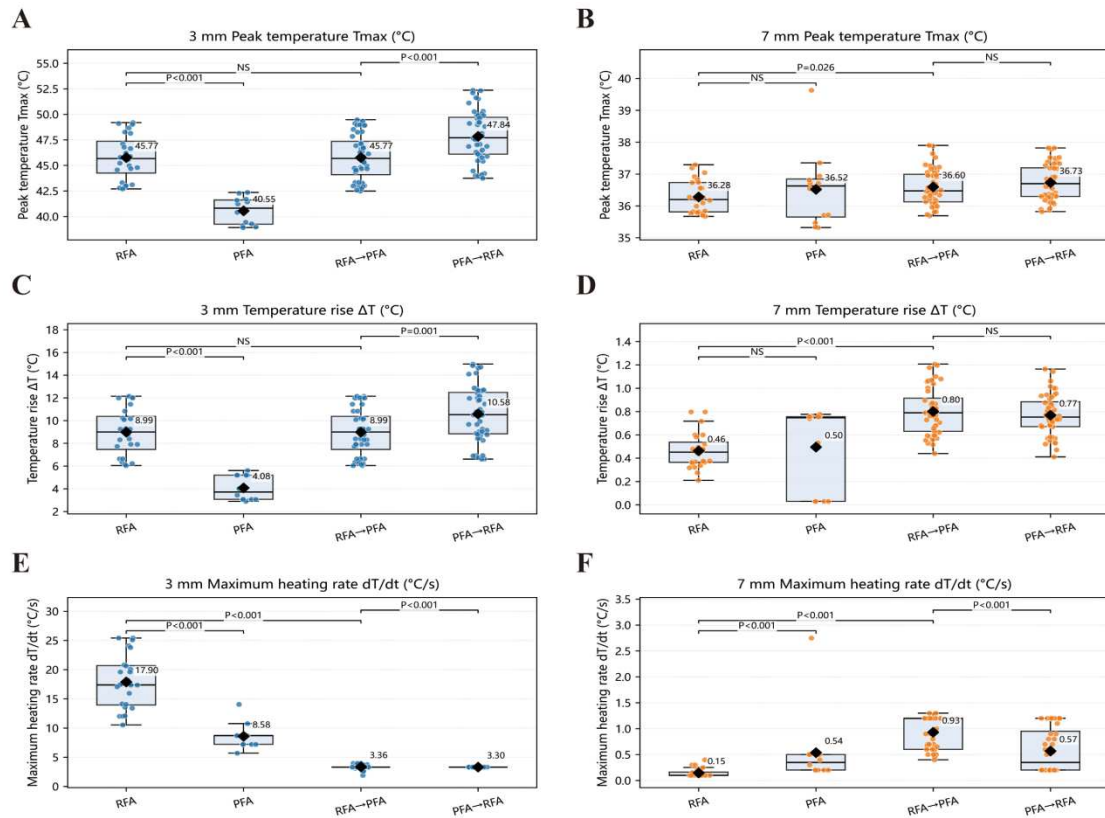


Fig. 5 Comparison of deep temperature safety indicators under different ablation modes A: 3 mm peak temperature Tmax; B: 7 mm peak temperature Tmax; C: 3 mm temperature rise ΔT; D: 7 mm temperature rise ΔT; E: 3 mm maximum heating rate dT/dt; F: 7 mm maximum heating rate dT/dt.

Intergroup Statistical Comparison of Temperature Safety Indicators

Further statistical analysis revealed significant intergroup differences in both peak temperature and temperature rise at 3 mm (Fig. 5A and C). For the 3 mm peak temperature Tmax, the PFA group was the lowest, with an average of approximately 40.55°C. The RFA group and RFA→PFA group were similar, with an average of approximately 45.77°C. The PFA→RFA group showed the highest value, with an average of approximately 47.84°C. Statistical comparison showed that the 3 mm Tmax values in the RFA, RFA→PFA, and PFA→RFA groups were all significantly higher than those in the PFA group, and the PFA→RFA group was further increased compared with the RFA→PFA group. The 3 mm temperature rise ΔT showed a similar trend. The PFA group had the lowest average ΔT, approximately 4.08°C. The RFA and RFA→PFA groups had an average of approximately 8.99°C, while the PFA→RFA group had an average of approximately 10.58°C, suggesting that the sequential mode of PFA followed by RFA could produce a more pronounced temperature rise at 3 mm.

By contrast, the temperature indicators at 7 mm changed only slightly across the four ablation

modes (Fig. 5B and D). For the 7 mm peak temperature $T_{\max}$, the RFA group averaged approximately 36.28°C, the PFA group approximately 36.52°C, the RFA→PFA group approximately 36.60°C, and the PFA→RFA group approximately 36.73°C. All were markedly lower than the $T_{\max}$ at 3 mm. For the 7 mm temperature rise ΔT , the RFA group averaged approximately 0.46°C, the PFA group approximately 0.50°C, the RFA→PFA group approximately 0.80°C, and the PFA→RFA group approximately 0.77°C. Although the 7 mm ΔT in the sequential dual-energy groups was slightly higher than that in the simple RFA group, the overall temperature rise remained less than 1°C, suggesting limited heat conduction to deeper tissue.

The maximum heating-rate analysis showed that the RFA group had the highest dT/dt at 3 mm, averaging approximately 17.90°C/s, whereas the PFA group ranked second at approximately 8.58°C/s (Fig. 5E). The RFA→PFA and PFA→RFA groups had markedly lower values, at approximately 3.36°C/s and 3.30°C/s, respectively. At 7 mm, the maximum heating rate was generally low: approximately 0.93°C/s in the RFA→PFA group, 0.57°C/s in the PFA→RFA group, 0.54°C/s in the PFA group, and 0.15°C/s in the RFA group (Fig. 5F). These results suggest that simple RFA showed a faster early heating characteristic at 3 mm, whereas sequential dual-energy ablation, although producing higher $T_{\max}$ and ΔT , showed a relatively slower heating process. The uniformly low rates at 7 mm further indicate that deep temperature changes were relatively slow.

The 7 mm-to-3 mm temperature-rise attenuation ratio remained far below 1 in every group (Fig. 6). The attenuation ratio was approximately 0.05 in the RFA group, approximately 0.11 in the PFA group, approximately 0.09 in the RFA→PFA group, and approximately 0.07 in the PFA→RFA group. These results indicate that, regardless of whether single-energy ablation or sequential dual-energy ablation was used, heat conduction from 3 mm to 7 mm was significantly attenuated. Although the attenuation ratio in the PFA group was relatively higher, its absolute temperature rise at 3 mm was low, and therefore this does not indicate a higher deep thermal risk. The RFA and sequential dual-energy groups produced a more evident temperature rise at 3 mm, but the temperature rise at 7 mm remained low, suggesting that the risk of deep heat conduction was generally limited.

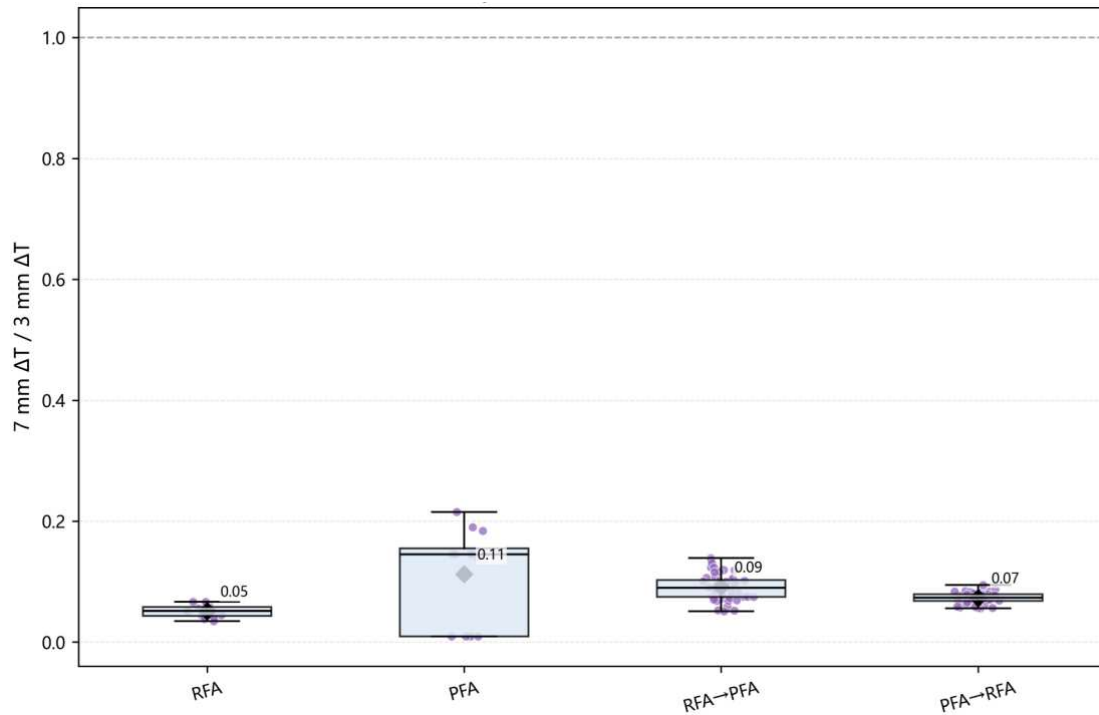


Fig. 6 Comparison of the 7 mm-to-3 mm temperature rise attenuation ratio

Taken together, Figures 2 to 6 show that the temperature response at 3 mm in the simple RFA group increased with increasing AI value, with AI=500 producing the most evident temperature rise. The simple PFA group showed an overall lower temperature response, and PFA 2× produced higher 3 mm T_{max} and ΔT than PFA 1×. In the sequential dual-energy groups, the PFA→RFA mode produced the highest T_{max} and ΔT at 3 mm, especially the PFA 2×+RFA AI=500 combination, which showed the most prominent temperature response. The RFA→PFA group showed a relatively higher maximum heating rate at 7 mm. Nevertheless, T_{max} , ΔT , and dT/dt at 7 mm were all markedly lower than those at 3 mm, and the 7 mm-to-3 mm temperature rise attenuation ratios were far below 1, indicating that deep heat conduction was clearly limited. Therefore, while sequential dual-energy ablation enhanced the temperature response at 3 mm and lesion formation efficacy, it did not cause obvious temperature accumulation at 7 mm. However, temperature dynamic differences remained between different energy sequences, and the ablation efficacy and thermal safety should be comprehensively evaluated in combination with TTC lesion size, HE staining, and TUNEL staining results.

Intragroup Comparison of TTC-Stained Lesion Size and Screening of Maximum Injury Parameters

RFA Group

TTC staining showed that different degrees of ablation injury were observed on myocardial sections in each group. The red-stained areas mainly represented relatively viable myocardium, whereas the white or pale-yellow areas mainly represented ablation injury. Across AI 350, 400, 450, and 500, the simple RFA group formed progressively different degrees of TTC-negative

lesions (Fig. 7A). As the AI value increased, the white or pale-yellow injury area on the sections gradually enlarged. The lesion area was smallest in the AI 350 group, gradually increased in the AI 400 and AI 450 groups, and was most evident in the AI 500 group, suggesting a positive correlation between RFA lesion size and AI value. Both lesion width and lesion depth increased gradually with increasing AI value. In terms of width, the AI 350 group showed the lowest value, followed by progressive increases in the AI 400, AI 450, and AI 500 groups. A similar trend was observed for lesion depth, with the AI 500 group showing the greatest lesion depth. These statistical results indicate that increasing the AI value can enhance the injury efficacy of RFA on myocardial tissue.

Taken together, the representative TTC images and quantitative results showed that AI 500 produced the greatest lesion width and depth in the simple RFA group, and was therefore identified as the maximum injury parameter for this group. Thus, AI 500 was selected as the representative parameter for the simple RFA group in the subsequent comparison of maximum injury conditions among the four groups.

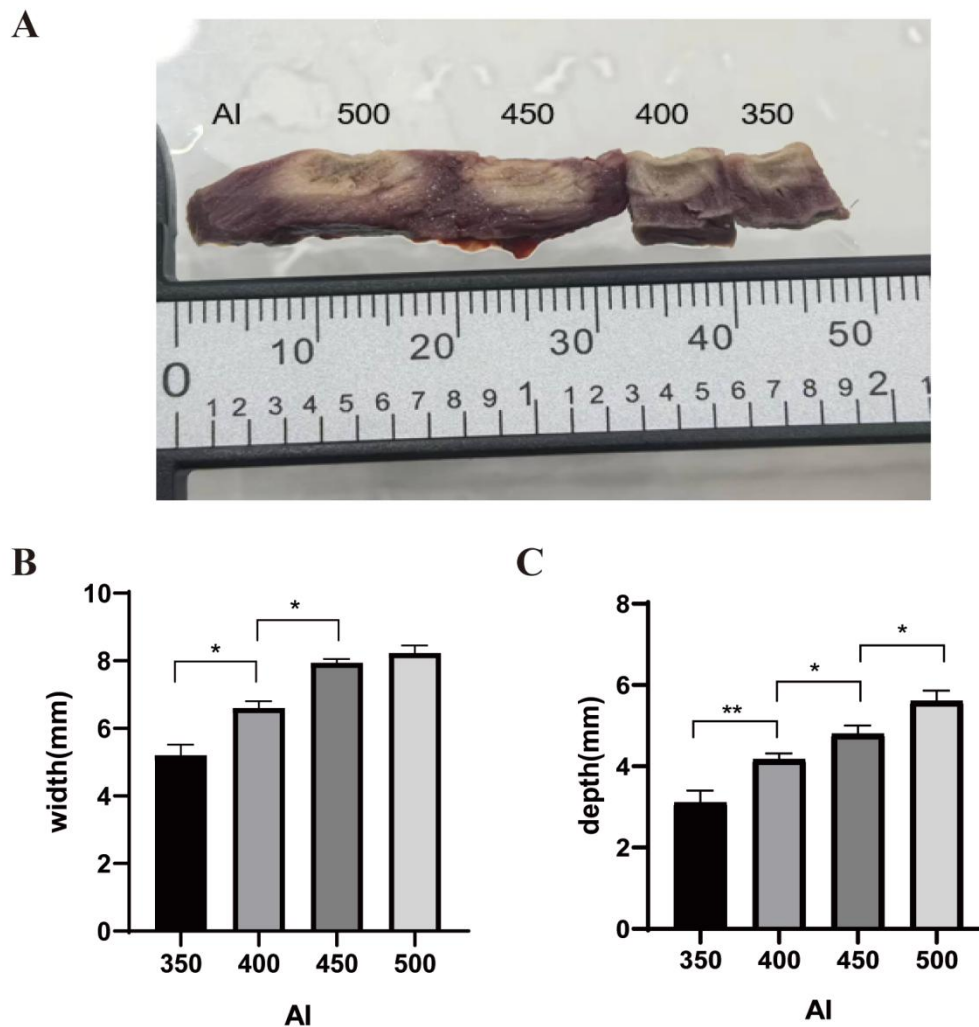


Fig. 7 TTC staining and comparison of lesion width and depth under different AI values in the

RFA group. A: Representative TTC staining images after RFA at AI 350, 400, 450, and 500; B: Comparison of lesion width among different AI groups; C: Comparison of lesion depth among different AI groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

PFA Group

In the simple PFA group, both one and two discharges produced localized pale lesions; however, the overall lesion size remained smaller than that in the high-AI RFA and sequential dual-energy groups (Fig. 8A). Compared with PFA 1 $\times$, the TTC-negative area after PFA 2 $\times$ was more evident, suggesting that increasing the number of pulsed field discharges could enhance local myocardial injury. Quantitative results showed that lesion depth was significantly greater in the PFA 2 $\times$ group than in the PFA 1 $\times$ group, and lesion width also showed an increasing trend. These results indicate that the injury efficacy of simple PFA was related to the number of discharges, and two discharges produced deeper myocardial injury than one discharge. Therefore, PFA 2 $\times$ was selected as the representative maximum injury parameter for the simple PFA group in the subsequent comparison of maximum injury conditions among the four groups

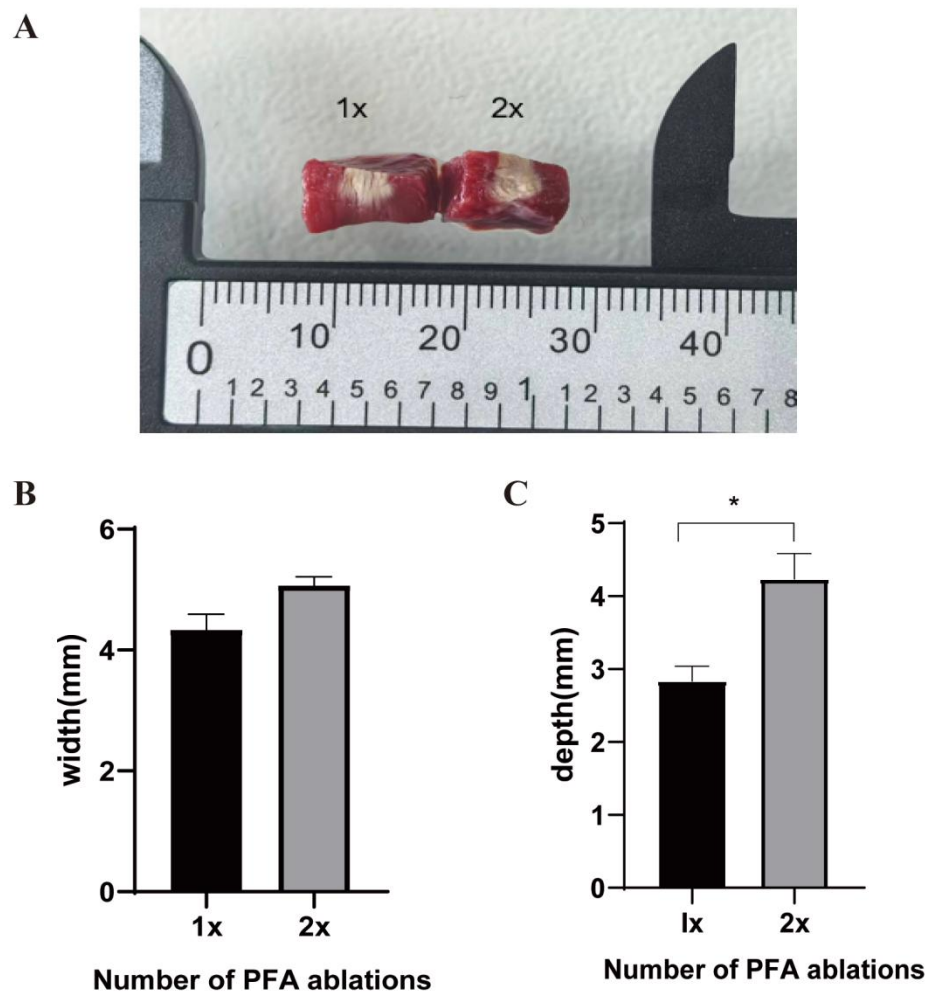


Fig. 8 TTC staining and comparison of lesion width and depth under different discharge

numbers in the PFA group. A: Representative TTC staining images after PFA 1× and PFA 2× discharge; B: Comparison of lesion width after PFA 1× and PFA 2× discharge; C: Comparison of lesion depth after PFA 1× and PFA 2× discharge. * $P < 0.05$.

RFA+PFA Group

In the RFA+PFA group, relatively clear TTC-negative lesions developed across the different RFA AI values followed by PFA discharge (Fig. 9A and B). Intragroup comparison showed that, under the same number of PFA discharges, lesion width and depth generally increased with increasing RFA AI value. Under the same AI value, PFA 2× produced a more evident lesion than PFA 1×, especially in terms of lesion depth. Among different parameter combinations, RFA AI 500 combined with PFA 2× produced the most evident TTC-negative area, and both lesion depth and width were relatively high. These results suggest that, in the sequential mode of RFA followed by PFA, adding two PFA discharges on the basis of high-AI RFA-induced thermal injury can further enlarge the myocardial injury area. Therefore, in the subsequent comparison of maximum injury conditions among the four groups, the RFA+PFA group specifically refers to the RFA AI 500+PFA 2× combination.

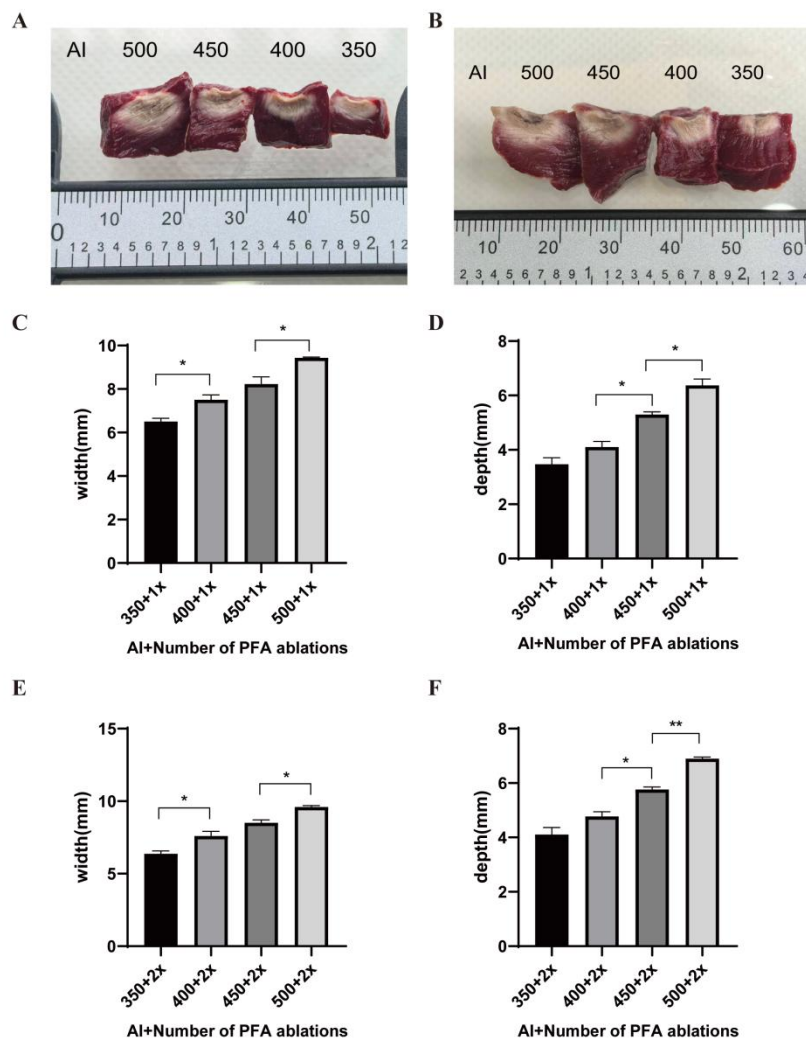


Fig. 9 TTC staining and comparison of lesion width and depth under different parameter combinations in the RFA+PFA group. A: Representative TTC staining images after different AI values of RFA followed by PFA 1×; B: Representative TTC staining images after different AI values of RFA followed by PFA 2×; C: Lesion width after different AI values of RFA+PFA 1×; D: Lesion depth after different AI values of RFA+PFA 1×; E: Lesion width after different AI values of RFA+PFA 2×; F: Lesion depth after different AI values of RFA+PFA 2×. * $P < 0.05$, ** $P < 0.01$.

PFA+RFA Group

PFA followed by RFA at different AI values likewise produced clearly visible pale-white TTC-negative lesions (Fig. 10A and B). Intragroup comparison showed that, under the same number of PFA discharges, lesion width and depth generally increased as the RFA AI value increased from 350 to 500. Under the same AI value, the lesion area after PFA 2× combined with RFA was generally larger than that after PFA 1× combined with RFA. Among different parameter combinations, PFA 2×+RFA AI 500 produced the most evident injury area, suggesting that, in the sequential mode of PFA followed by RFA, increasing the number of PFA discharges and combining them with high-AI radiofrequency energy can enhance lesion formation. Therefore, in the subsequent comparison of maximum injury conditions among the four groups, the PFA+RFA group specifically refers to the PFA 2×+RFA AI 500 combination

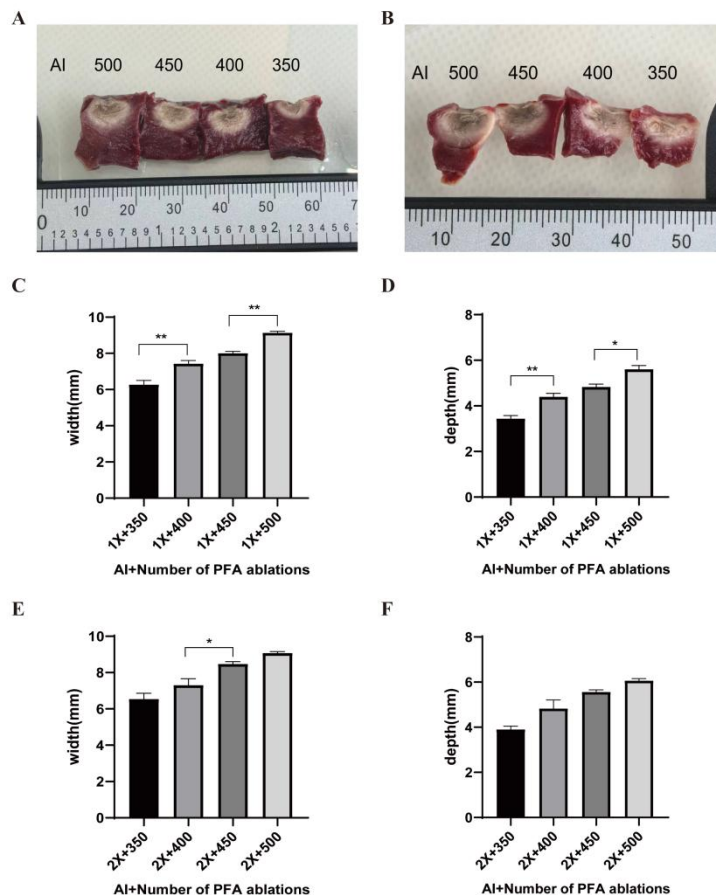


Fig. 10 TTC staining and comparison of lesion width and depth under different parameter combinations in the PFA+RFA group. A: Representative TTC staining images after PFA 1× followed by different AI values of RFA; B: Representative TTC staining images after PFA 2× followed by different AI values of RFA; C: Lesion width after PFA 1×+different AI values of RFA; D: Lesion depth after PFA 1×+different AI values of RFA; E: Lesion width after PFA 2×+different AI values of RFA; F: Lesion depth after PFA 2×+different AI values of RFA. *P < 0.05, **P < 0.01.

Intergroup Comparison of TTC Lesion Size Under Maximum Injury Parameters

After completing the intragroup parameter screening, the maximum injury condition of each group was selected for transverse comparison (Fig. 11), namely AI 500 for the simple RFA group, PFA 2× for the simple PFA group, RFA AI 500+PFA 2× for the RFA+PFA group, and PFA 2×+RFA AI 500 for the PFA+RFA group. The results showed that lesion width and depth were both relatively small in the simple PFA 2× group (width approximately 5.2 mm, depth approximately 4.3 mm), whereas the lesion size in the simple RFA AI 500 group was markedly greater than that in the simple PFA 2× group (width approximately 8.2 mm, depth approximately 5.7 mm), indicating that, under the present experimental conditions, the lesion formation efficacy of simple RFA was stronger than that of simple PFA. Compared with the single-energy groups, both sequential dual-energy groups further increased the TTC-negative injury area, suggesting that the combined application of RFA and PFA can enhance myocardial injury. Among them, the RFA+PFA 500+2× group showed the most prominent lesion depth (approximately 6.9 mm) and width (approximately 9.7 mm), indicating that the sequential mode of RFA followed by PFA may be more conducive to forming deep myocardial injury. The PFA+RFA 2×+500 group also produced a relatively large lesion (width approximately 9.2 mm, depth approximately 6.1 mm), but its deep extension was slightly lower than that of the RFA+PFA 500+2× group.

In summary, TTC staining showed that AI 500 produced the greatest injury in the simple RFA group, while two discharges produced the greatest injury in the simple PFA group. In the sequential dual-energy groups, the combination of RFA AI 500 and PFA 2× represented the maximum injury parameter under each sequence. Further comparison of the four maximum injury conditions showed that the RFA+PFA 500+2× group had the most prominent lesion depth, suggesting that the sequential ablation strategy of radiofrequency followed by pulsed field may have stronger deep injury efficacy.

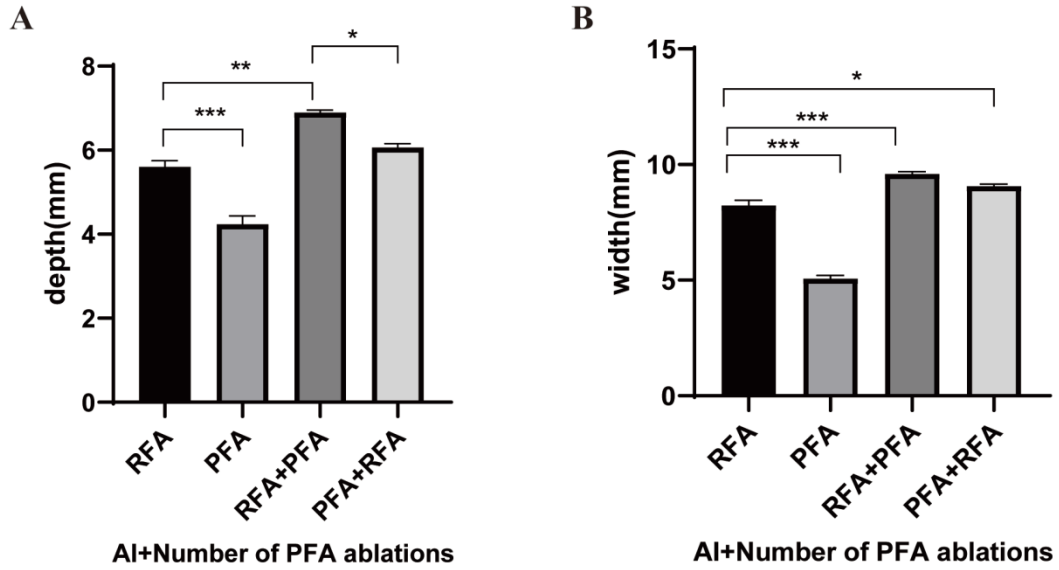


Fig. 11 Comparison of TTC-stained lesion width and depth under the maximum injury parameters of each group. A: Comparison of lesion width among the RFA AI 500, PFA 2×, RFA+PFA 500+2×, and PFA+RFA 2×+500 groups; B: Comparison of lesion depth among the four groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Histological Injury Features Observed by HE and TUNEL Staining

Based on the TTC staining results, representative myocardial samples obtained under the maximum-injury condition in each group were selected for HE and TUNEL staining, including RFA AI 500, PFA 2×, RFA AI 500+PFA 2×, and PFA 2×+RFA AI 500. HE staining was used to compare myocardial tissue architecture and cellular morphological changes, whereas TUNEL staining was used to evaluate DNA fragmentation and apoptosis-like cell death under the different ablation modes. These complementary assessments are illustrated by the representative HE-stained morphology in Fig. 12 and the TUNEL findings in Fig. 13.

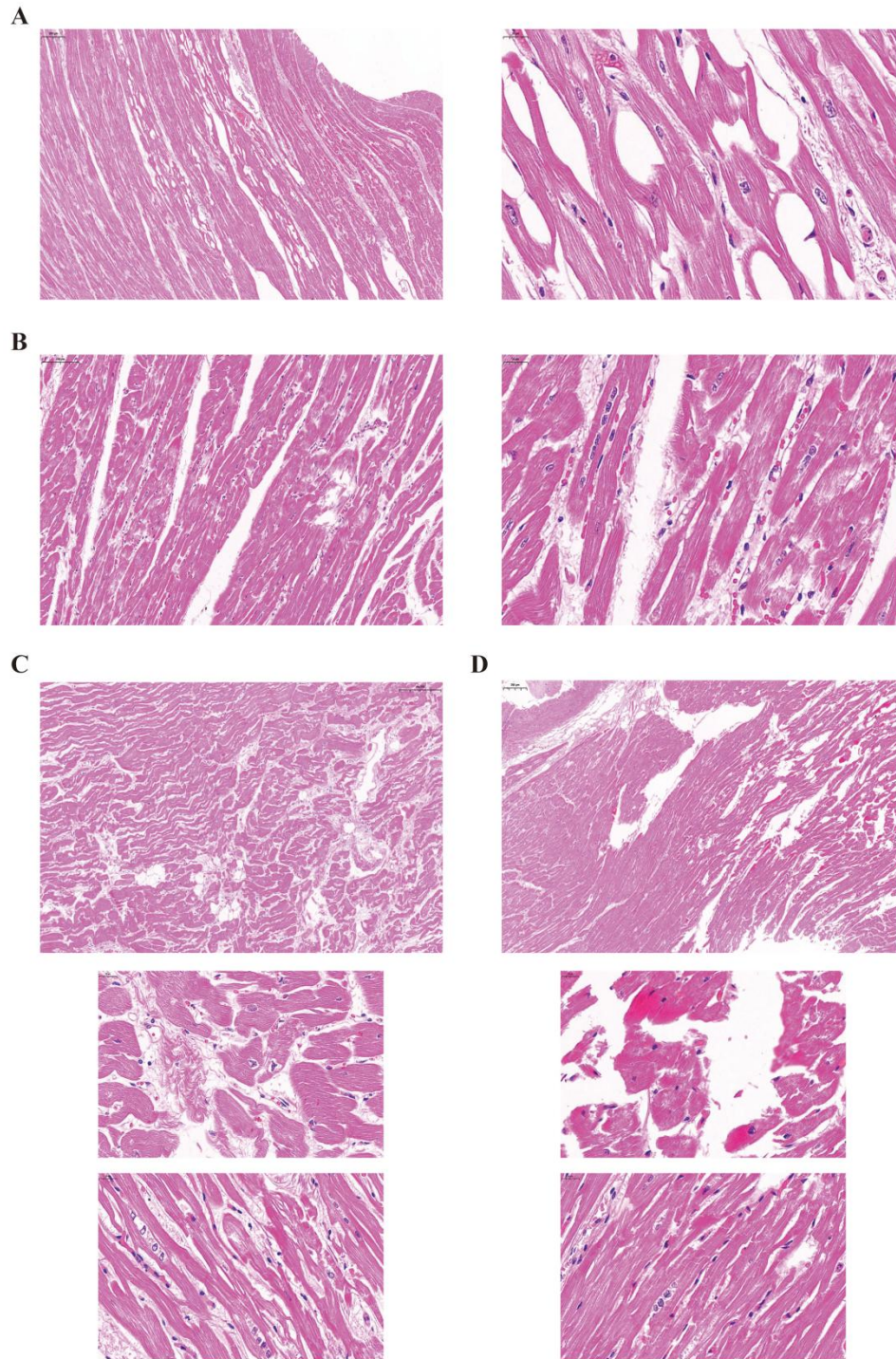


Fig. 12 Representative HE-stained histological morphology of myocardial tissue under the maximum-injury parameters of the four ablation groups. (A) RFA AI 500 group, 200 μm field; (B) PFA 2 $\times$ group, 200 μm field; (C) RFA AI 500 group, 20 μm field; (D) PFA 2 $\times$ group, 20 μm field; (E) RFA AI 500+PFA 2 $\times$ group, 200 μm field; (F) PFA 2 $\times$ +RFA AI 500 group, 200 μm field; (G, H) representative 20 μm fields of the RFA AI 500+PFA 2 $\times$ group; and (I, J) representative 20 μm fields of the PFA 2 $\times$ +RFA AI 500 group. Scale: 200 μm in panels A, B, E, and F; 20 μm in panels C, D, and G–J.

HE staining revealed distinct histomorphological injury patterns among the four ablation groups (Fig. 12). In the RFA AI 500 group (Fig. 12A and C), the myocardial tissue mainly exhibited typical thermal coagulative necrosis, characterized by cardiomyocyte swelling, irregular cellular morphology, nuclear dissolution or disappearance, cell membrane disruption, and relatively homogeneous tissue damage. In contrast, the PFA 2× group (Fig. 12B and D) predominantly showed electroporation-related injury, including cell shrinkage, nuclear pyknosis, chromatin condensation, and relatively preserved cell membranes, consistent with apoptosis-like morphological changes.

Both sequential dual-energy groups exhibited a coexistence of necrotic and apoptosis-like changes, although the predominant injury pattern differed according to the sequence of energy delivery. In the RFA AI 500+PFA 2× group (Fig. 12E, G, and H), necrotic injury predominated, with pronounced disruption of myocardial architecture, cardiomyocyte swelling, nuclear dissolution, and extensive necrotic areas. The boundary of apoptosis-like injury was poorly defined, suggesting that when RFA was delivered before PFA, thermal injury remained the dominant mechanism, whereas the subsequent PFA application mainly extended the overall area of tissue injury.

In the PFA 2×+RFA AI 500 group (Fig. 12F, I, and J), myocardial necrosis was also observed, but apoptosis-like morphological features were partially retained, including nuclear pyknosis, chromatin condensation, and a relatively distinct boundary of apoptosis-like injury. These findings suggest that when PFA was delivered before RFA, PFA-induced electroporation and apoptosis-like injury coexisted with the thermal necrosis subsequently produced by RFA.

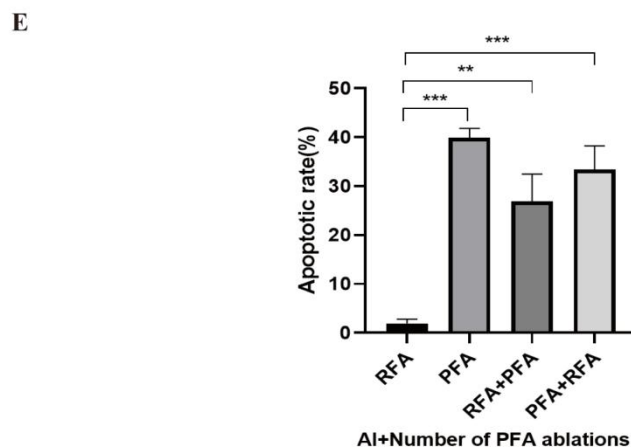
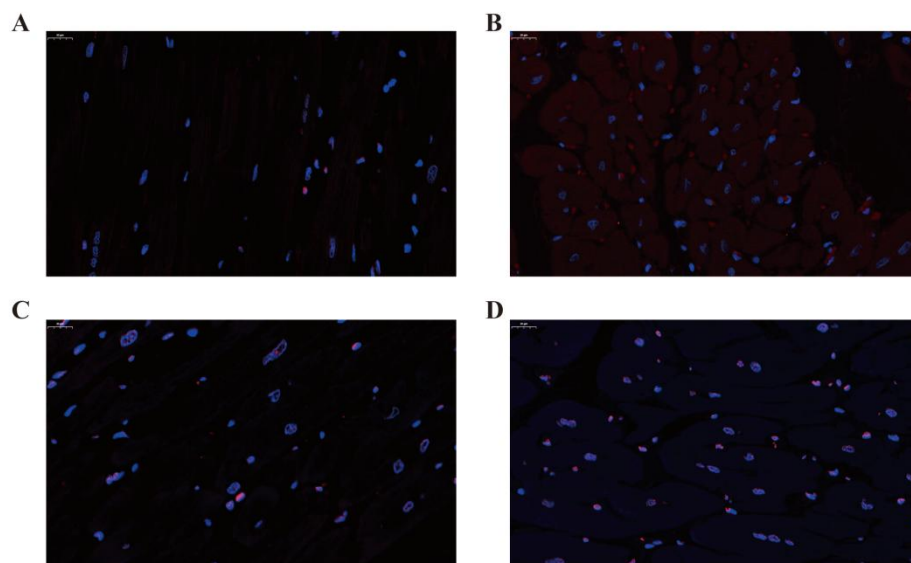


Fig. 13 Representative TUNEL staining and quantitative comparison of TUNEL-positive cell rates under the maximum-injury parameters of the four ablation groups. (A) RFA AI 500 group; (B) PFA 2× group; (C) RFA AI 500+PFA 2× group; (D) PFA 2×+RFA AI 500 group; and (E) quantitative comparison of TUNEL-positive cell rates among the four groups. Panels A–D show representative fluorescence images, in which blue fluorescence indicates cell nuclei and red fluorescence indicates TUNEL-positive signals. Data in panel E are presented as mean ± standard deviation. **P < 0.01 and ***P < 0.001 compared with the RFA AI 500 group.

TUNEL staining revealed different degrees of positive signals in the ablated myocardial tissue among the four groups (Fig. 13A–D). The RFA AI 500 group exhibited only sparse TUNEL-positive signals, whereas the PFA 2× group showed markedly increased red fluorescent signals. The RFA AI 500+PFA 2× and PFA 2×+RFA AI 500 groups also displayed evident TUNEL-positive signals, although their distributions and intensities differed between the two energy sequences.

Quantitative analysis further demonstrated significant differences in the TUNEL-positive cell rate among the four ablation groups (Fig. 13E). The RFA AI 500 group had the lowest TUNEL-positive cell rate, at approximately 2%. Compared with the RFA AI 500 group, the TUNEL-positive cell rate was significantly higher in the PFA 2× group, at approximately 40% (P < 0.001), in the RFA AI 500+PFA 2× group, at approximately 27% (P < 0.01), and in the PFA 2×+RFA AI 500 group, at approximately 33% (P < 0.001). Among the four groups, the PFA 2× group showed the highest TUNEL-positive cell rate, whereas the two sequential dual-energy groups exhibited intermediate levels.

Combined with the HE staining findings, the limited TUNEL positivity in the RFA AI 500 group indicates that myocardial cell death after RFA was predominantly caused by thermal coagulative necrosis. In contrast, the strong TUNEL-positive signals and the substantially increased positive cell rate in the PFA 2× group were consistent with nuclear pyknosis and chromatin condensation observed by HE staining, suggesting that PFA was more likely to induce electroporation-related apoptosis-like cell death.

The RFA AI 500+PFA 2× group showed a significantly higher TUNEL-positive cell rate than the RFA AI 500 group but remained morphologically dominated by necrotic injury. This finding suggests that when RFA was performed before PFA, thermal coagulative necrosis remained the principal injury mechanism, while subsequent PFA increased DNA fragmentation and apoptosis-like cellular injury. The PFA 2×+RFA AI 500 group showed a relatively high TUNEL-positive cell rate together with necrotic morphological changes, indicating the coexistence of PFA-induced apoptosis-like injury and subsequent RFA-induced thermal necrosis.

Overall, simple RFA predominantly produced thermal coagulative necrosis, whereas simple PFA generated the strongest TUNEL-positive response and was mainly associated with electroporation-related apoptosis-like injury. Both sequential dual-energy groups showed mixed patterns of necrosis and apoptosis-like cell death. However, RFA followed by PFA remained more strongly characterized by necrosis, whereas PFA followed by RFA retained a greater degree of apoptosis-like injury. These findings indicate that the sequence of RFA and

PFA affects not only lesion size but also the relative contribution of different myocardial cell-death mechanisms.

Discussion

In this ex vivo study, we compared lesion formation, deep tissue temperature, and histological injury between RFA, PFA, and their sequential combinations in porcine myocardium. Sequential dual-energy ablation produced deeper lesions—most clearly in the RFA AI 500 + PFA 2× group—while temperature at 7 mm changed very little, indicating that deep thermal conduction remains limited even when two energy sources are stacked. These findings lend preclinical support to the dual-energy approach currently being tested in patients [37–39].

Depth Advantage of Dual-Energy Ablation

For lesions to be transmural—especially across the left atrial posterior wall or ventricular scar—depth is the main determinant of durable efficacy. RFA creates consistent thermal coagulative necrosis, but impedance rise, steam pops, and collateral damage to the esophagus and phrenic nerve limit how safely it can be delivered [1, 2]. PFA works through irreversible electroporation in a tissue-selective, non-thermal way, but a single application yields fairly shallow lesions, and whether those lesions hold up in thick tissue is still debated [3–5].

We found that RFA lesion dimensions tracked AI in a dose-dependent fashion, and adding a second PFA discharge enlarged lesions beyond what a single discharge produced. Both sequential groups outperformed PFA alone, which suggests that thermal coagulation and electroporation complement each other rather than simply adding up. The order of delivery made a difference: RFA before PFA gave the deepest lesions, consistent with recent preclinical work showing that warming target tissue lowers the electric-field threshold for irreversible electroporation [30, 40]. Raising tissue temperature from 20 °C to 37 °C can lower that threshold by about 40% and increase ablation volume by more than 20% [40–42].

Early clinical data point in the same direction. Reddy et al. reported 100% acute pulmonary vein isolation using a lattice-tip dual-energy catheter, with 97% durable isolation at 3 months after waveform optimization [37]. In the SmartFIRE trial, dual-energy focal ablation achieved 87% durable isolation at 3 months, and post-procedural endoscopy showed no esophageal lesions [38]. A recent randomized trial in persistent atrial fibrillation found dual-energy ablation superior to conventional RFA for 1-year effectiveness [39]. Our ex vivo findings help explain these results: they show how sequence-dependent thermal preconditioning produces deeper lesions.

Thermal Safety of Dual-Energy Superposition

Delivering extra energy to the posterior wall immediately brings up the risk of atrioesophageal fistula—the most feared complication of atrial fibrillation ablation [2]. We therefore tracked deep tissue temperature at 3 mm and 7 mm throughout. Every group that included RFA showed a measurable rise at 3 mm; the PFA→RFA group tended toward higher T_{max} and ΔT, probably because electroporation changes local tissue impedance and redirects subsequent RF current [16]. At 7 mm, by contrast, T_{max} and ΔT remained low in all groups, and the 7 mm-to-3 mm

attenuation ratios stayed well below 1. In short, surface heat did not build up at depth.

This fits with the low esophageal injury rates seen in clinical series. The SmartfIRE trial found no esophageal lesions in 31 patients who underwent endoscopy [38], and preclinical studies have confirmed that PFA damages cardiomyocytes while sparing esophageal smooth muscle and nerve tissue [43–45]. That said, PFA is not entirely athermal: repeated discharges do produce measurable Joule heating, as our data and others' have shown [10, 15]. In our experiments, PFA 2× raised 3 mm temperature by a mean of 4.08 °C—some heating clearly occurs, but it is far less than with RFA and drops off quickly with depth.

The thermal footprint of PFA, though measurable, is distributed differently from that of RFA. RFA generates sustained, radial heat diffusion that can travel toward deeper structures, whereas PFA-related Joule heating stays mostly at the electrode-tissue interface and fades rapidly between pulses. This difference may help explain why even sequential PFA-containing protocols did not cause dangerous temperature build-up at 7 mm in our model, although the lack of perfusion in ex vivo tissue means these data should be read with that caveat in mind [10, 15].

Effect of Energy Sequence on Injury Mechanism

HE and TUNEL staining showed different injury patterns depending on which energy was used and in what order. RFA alone produced cell swelling, nuclear dissolution, and loss of architectural detail—the typical appearance of thermal coagulative necrosis [32, 33]. PFA alone caused cell shrinkage, nuclear pyknosis, and chromatin condensation, in keeping with electroporation-driven apoptosis-like cell death. This pattern—TUNEL positivity peaking around 24 hours, then gradual fibrotic replacement—has been described in earlier PFA studies [46, 47]. The tissue selectivity of PFA, with relative sparing of vessels and nerves, probably arises from this mechanism, which is fundamentally unlike the non-specific thermal destruction caused by RFA [48–50].

In the sequential groups, which cell death pathway dominated depended on the order. When RFA was delivered first, necrosis overshadowed any apoptotic response, which suggests that thermal injury enlarged the lesion core and that subsequent PFA mainly extended the injury margin through thermally lowered electroporation thresholds. When PFA came first, both necrosis and apoptosis-like changes were seen, indicating that electroporation and the following thermal injury acted in parallel rather than in sequence. How these different cell death profiles affect chronic lesion healing and arrhythmogenicity is still unknown, although clinical data suggest that PFA lesions may heal with more homogeneous fibrosis and less inflammation than RFA lesions [44]. To characterize how dual-energy lesions heal, chronic animal models will be needed.

This shift in cell death mechanism according to delivery sequence has not, to our knowledge, been reported before. If necrosis-dominant lesions (RFA+PFA) heal with greater heterogeneity than those that retain apoptosis-like features (PFA+RFA), the resulting scar may also differ in arrhythmogenic potential. We lack chronic healing data for dual-energy lesions, but earlier work comparing PFA and RFA scar morphology offers a reasonable starting point: PFA lesions tend to heal with more homogeneous fibrosis and less inflammatory infiltration [44], and the same may hold for the PFA-first sequence in our study. Answering this question will require

survival studies with histological assessment at several time points.

Limitations

Several limitations should be noted. First, the ex vivo porcine heart model has no coronary perfusion or convective heat-sink from circulating blood, which may overestimate both lesion size and temperature rise; coronary flow is known to substantially attenuate thermal injury [30]. Second, inserting PT100 probes may have created small channels for heat conduction, although the fine-gauge design (1.5 mm) was chosen to limit this artifact. Third, we assessed only acute lesions—chronic healing, transmural, and arrhythmogenic potential cannot be inferred from these data. Fourth, RFA and PFA used different catheter geometries (linear vs. circular), so electrode-tissue contact differences may have introduced confounding. Finally, we did not include electrophysiological endpoints such as conduction block and impedance change.

In vivo studies with chronic survival endpoints, esophageal temperature monitoring, and electrophysiological testing will be needed to confirm these findings and to refine dual-energy ablation protocols.

Conclusions

Sequential RFA and PFA ablation in this ex vivo model produced deeper myocardial lesions than either modality alone, while heat conduction to 7 mm stayed limited—supporting an acceptable deep thermal safety margin. The order of energy delivery determined not just lesion depth but also the dominant cell death mechanism: RFA+PFA leaned toward thermal necrosis, whereas PFA+RFA retained a component of apoptosis-like injury. For clinical translation, especially for posterior wall ablation, an RFA-first approach appears to offer the best balance of depth and thermal safety. Further work to optimize energy sequence and dosing will be important for extending this approach to the ablation of complex arrhythmias.

Abbreviations

Abbreviation	Definition
RFA	Radiofrequency ablation
PFA	Pulsed field ablation
AI	Ablation index
TTC	Triphenyltetrazolium chloride
HE	Hematoxylin and eosin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
PBS	Phosphate-buffered saline
ANOVA	Analysis of variance
IACUC	Institutional Animal Care and Use Committee
ΔT	Temperature rise
dT/dt	Maximum heating rate

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Authors' contributions

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Data availability

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

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of University of Science and Technology of China. All procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹First Affiliated Hospital of University of Science and Technology of China, Hefei, China.

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