Radiofrequency Ablation Does Not Induce Apoptosis in the Rat Myocardium

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Background: *The mechanisms implicated in the genesis of delayed radiofrequency (RF) effects remain unclear, but may be related to extension of the lesion beyond the region of coagulative necrosis. The role of apoptosis in this process has not been previously reported. We assessed whether RF promotes apoptosis in the region surrounding acute ablation lesions in a rat model.*

Methods: *Wistar rats (n = 30; weight 300 g) were anesthesized, the chest was opened, and the heart was exposed. A modified unipolar RF ablation (custom catheter 4.5-mm-tip diameter, 12 Watts, 10 seconds) was undertaken on the left ventricular anterolateral epicardial surface and the chest was closed. After 2 hours, animals were killed for histological (hematoxylin and eosin, TdT-mediated dUTP Nick End-Labeling [TUNEL] assay) and immunohistochemical (anti-BAD and anti-caspase 3 antibodies) analysis (n = 18). Additional animals (n = 12) were sacrificed at 2 (n = 3), 24 (n = 3), 48 (n = 3), and 72 hours (n = 3) after ablation exclusively for anti-BAD Western Blotting analysis.*

Results: *Lesions were characterized by well-defined regions of coagulative necrosis. In 18/18 (100%) animals, TUNEL assay revealed positive luminescent reaction cells in the region surrounding the lesion, extending up to 2 mm from the border zone. However, microscopic evaluation of the nuclei and immunohistochemical and anti-BAD Western Blotting analysis were negative in all (100%) rats. Thus, positive TUNEL reaction in the periphery of the ablation lesion likely reflects nonspecific DNA damage.*

Conclusion: *RF ablation does not promote apoptosis in the periphery of the myocardial lesion. This finding may have implications for the elucidation of late lesion extension following RF ablation. (PACE 2012; 35:449–455)*

arrhythmias, radiofrequency, late effects, apoptosis, myocardium, rats

Introduction

Radiofrequency (RF) ablation has been extensively used for the curative treatment of cardiac arrhythmias such as atrioventricular (AV) nodal reentrant tachycardia and arrhythmias involving accessory AV connections.[1] However, as the technique became more widespread, late adverse effects of RF were recognized, especially regarding ablation in regions close to the conduction system, where AV blocks may occur seconds to months after the procedure.[2,3]

The mechanisms responsible for the delayed effects of RF are still uncertain, but seem to be related to the extension of the lesion beyond the area of acute coagulative necrosis. This process may be secondary to the inflammatory response, resulting in the extension of fibrosis to adjacent areas, injury to the microcirculation, and/or ultrastructural damage to the myocardium that surrounds the lesion.[3–6]

RF damage is essentially thermally mediated, resulting in coagulative tissue necrosis. Since during the application of RF energy the heating of tissue is reduced with increasing distance of the catheter tip, cells of regions peripheral to the necrotic area may undergo insufficient heating to cause acute necrosis, but sufficient to provoke cell damage.[3–6] In this area of transition, many cells fully recover, but others progress to necrosis and later fibrosis. Another possibility is that these cells will eventually develop programmed cell death, so called apoptosis.[7–14] Whereas the death process occurring in necrosis is abrupt, with damage to vital structures such as plasma and nuclear membranes, the process occurring in apoptosis is programmed.[13]

Apoptosis activates a cascade of cellular pathways that lead to DNA fragmentation, cellular involution, and phagocytosis.[7,8] Since in this process the cell membrane remains intact, there is no extravasation of the cytosol into the

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intercellular medium, a factor that explains the absence of inflammation in this process of cell death.[10]

The presence of apoptosis at the periphery of the RF ablation lesion has been reported both in hepatic tissue and in the myocardium of pigs.[15,16] However, these findings were based on the TUNEL (TdT-mediated dUTP Nick End-Labeling) technique, which is known to be imprecise since it may reflect nonspecific DNA damage.[17] Thus, it is still unsettled whether RF ablation promotes apoptosis in the myocardium.

The objective of this study was to determine the occurrence of acute apoptosis at the periphery of lesions generated by RF ablation on the free ventricular wall of rats.

Methods

The study was approved by the Research Ethics Committee of the Federal University of São Paulo and was conducted according to institutional norms.

Experimental Design and Animal Preparation

Thirty healthy male Wistar rats aged 7–8 weeks and weighing 288–310 g were used. After inhalatory halothane anesthesia, the animals were intubated and ventilated under positive pressure with a ventilator for rodents (Harvard Apparatus 683, Holliston, MS, USA).

Ablation Protocol

After thoracotomy in the 4th intercostal space, the heart of the animals was exposed and suspended with a customized clamp (also used as an indifferent electrode) and ablation was performed with a customized catheter with an electrode 4.5 mm in diameter (Fig. 1), using a TEB RF10 RF generator (Tecnologia Eletrônica Brasileira, São Paulo, Brazil). One ablation per animal was performed on the free wall of the left ventricle with a unipolar RF current (fixed 12 Watts power for 10 seconds) according to a technique developed and previously described by our group.[18] These RF settings produce sizable left ventricular lesions without inducing severe acute heart failure.

During each application, only impedance (in Ω) was monitored and the final value was recorded for analysis since the catheter does not have a temperature sensor. Next, the heart was rapidly placed back inside the thorax, pulmonar hyperinsufflation was performed, and the chest was closed with simple suture. The procedures were performed under sterile conditions and with antiseptic techniques in order to prevent infection.

Postablation Period

Eighteen animals were sacrificed 2 hours after ablation for macroscopic and microscopic evaluation and analysis by the TUNEL technique and also by immunohistochemistry. Additional animals with a longer follow-up time (n = 12) were sacrificed at each time point studied, that is, 2 hours (n = 3), 24 hours (n = 3), 48 hours (n = 3), and 72 hours (n = 3) after ablation exclusively for Western Blotting analysis.

Macroscopic Analysis

After removal of the heart from the 18 animals, the surface of the lesion was measured on the longitudinal and transverse axes with a millimeter ruler. A thin slice of the heart was then cut transversely to the center of the lesion and incubated in 1% triphenyl tetrazolium chloride for 7 minutes. The depth and the area of the lesion were determined using the ImageTool® software (version 3.0, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA) as shown in Figure 2. The following regions were standardized for analysis: (1) region n° 1, corresponding to the ablated region; (2) region n° 2, corresponding to the transition zone between ablated and healthy tissue; (3) region n° 3, healthy and located at a distance from the ablated region.

Microscopic Analysis

The lesions were cut transversely to their center into various histological sections so as to permit a good distinction between the necrotic

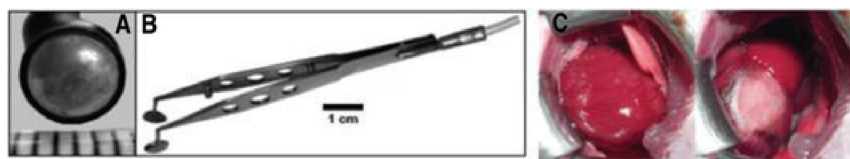

Figure 1. *Customized instruments for ablation in rats. (A) Tip of the unipolar catheter 4.5 mm in diameter used for ablation. (B) Clamp used to suspend the heart and also used as the indifferent electrode in ablation. (C) Image of the heart before and immediately after ablation. Note the easily delimited circular whitish lesion.*

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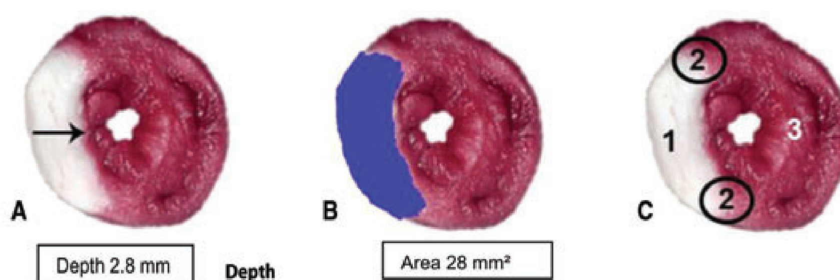

Figure 2. *Transverse section of the heart stained with tetrazolium illustrating the dimensions of the left ventricular free wall lesion. (A) Analysis of lesion depth (mm). (B) Analysis of the segmental area of the lesion measured with the Image Tool software (mm^2). (C) Regions analyzed: 1, ablated area; 2, transition zone; 3, healthy tissue. See text for details.*

and healthy areas. The slides were stained with hematoxylin and eosin (HE) and sent to two experienced pathologists for qualitative analysis of the lesions (n = 13).

Investigation of Apoptosis

To determine the presence of apoptosis at the periphery of the RF lesion (area 2), material from 18 animals was analyzed by the TUNEL assay[16] and by immunohistochemistry for BAD and caspase.[17] In 13/18 lesions, microscopic evaluation of the nuclei was performed by two experienced pathologists.[12] To validate the presence of apoptosis, a positive TUNEL was required, associated with at least one of the following criteria: BAD, caspase, or nuclear morphology compatible with apoptosis.

Western Blotting for the BAD protein[17] was performed in 12 additional animals sequentially sacrificed 2, 24, 48, and 72 hours after ablation (n = 3 at each time point) in order to determine whether apoptosis occurred after a longer follow-up time, as reported by Rai et al.[15] in a study on hepatic tissue ablation.

TUNEL Assay

The test was carried out with emphasis on the peripheral zone of the lesion, that is, region 2, using silanized paraffin-embedded tissue slides. After antigen recovery in a pressure pot for 20 minutes at approximately 120°C, the assay was carried out according to the instructions of the *in situ* Roche Science® kit (Indianapolis, IN, USA) for the detection of cell death.

Immunohistochemistry

The paraffin-embedded tissue blocks were cut into 4-μm-thick sections and fixed in 10% formaldehyde, and peroxidase activity was blocked with H_2O_2 for 15 minutes. Rabbit anti-BAD monoclonal antibody (1:100, Abcam®, Cambridge, UK) and anti-caspase monoclonal antibody (1:200, Abcam) were used as primary antibodies. Primary incubation was carried out for 18 hours and a biotin conjugate diluted at 1:400 (Dako, Ely, UK) for 30 minutes at 37°C was used as secondary antibody. An avidin-biotin-peroxidase complex (Vector Lab, Burlingame, CA, USA) was added and consecutively incubated for 20 minutes. A negative control reaction was carried out by omitting the primary antibody and a positive control tonsil slide was used to validate the reaction. The slides were examined under a microscope with a coupled camera and the images were captured and transmitted to the Leica QWin software (Leica Microsystems Inc., Buffalo Grove, IL, USA).

Protein Extraction and Western Blotting

Frozen cardiac tissue close to the ablation area was homogenized in cell lysis buffer (100-mM Tris, 50-mM NaCl, 10-mM EDTA, and 1% Triton X-100) with a protease inhibitor cocktail (Sigma Chemical Corp., St. Louis, MO, USA). Samples containing 30 μg of the homogenate were submitted to 10% SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences, Piscataway, NJ, USA) and the efficacy of transfer was monitored with 0.5% Ponceau S. The membrane was incubated with blocking buffer (5% skim milk, 10-mM Tris-HCl, pH 7.6, 150-mM NaCl, and 0.1% Tween 20) for 2 hours at room temperature and incubated overnight at 4°C with anti-BAD antibody at 1:500 dilution (Abcam). After incubation, the membranes were washed three times and then incubated for 1 hour at room temperature with a secondary rabbit antibody (Millipore, Billerica, MA, USA). The determinations were normalized with glyceraldehyde-3-phosphate dehydrogenase labeling.

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Morphological Criteria

For the histology protocol, we searched for cardiomyocyte nuclei present 2 mm after the transition area, in region 2. The objective of the study was to investigate nuclei with evidence of apoptosis, such as peripheral condensation of nuclear chromatin forming half-moons or fragmentation of the nucleus into strongly basophilic particles (apoptotic bodies).[12,17]

Statistical Analysis

Data are reported as means ± standard deviation. The categorical variables were analyzed by the χ^2 test and the continuous variables by the Student's *t*-test. The level of significance was set at $P < 0.05$.

Results

RF Ablation

One ablation per animal was performed (n = 30) using a fixed power of 12 Watts and a time of 10 seconds. Eight animals (26%) manifested ventricular fibrillation immediately after application, although the rhythm was reestablished with a brief mechanical stimulus with a clamp.

Macroscopic Analysis

The lesions were easily visualized macroscopically, usually being white, of circular morphology with defined margins, and closely similar in all 18 animals (Fig. 1C). The measurements of the transverse and longitudinal axes, of depth and area, were, respectively, 9.4 ± 0.5 mm (8.0–10.0 mm), 9.4 ± 0.6 mm (8.0–10 mm), 2.9 ± 0.2 mm (2.4–3.4 mm), and 27 ± 3 mm^2 (21–31 mm^2). The lesions were highly reproducible and of closely similar dimensions, with low standard deviations of the parameters analyzed.

Histological Analysis

In this stage, 13 animals were used and sacrificed 2 hours after ablation. Histological evaluation of the lesion revealed coagulative necrosis with interstitial hemorrhage of irregular distribution, cytoplasmic vacuolization, edema, and absence of an inflammatory infiltrate in all animals, in a highly homogeneous manner mainly in region 1. In region 2 it was possible to identify the transition between the ablated area and the healthy one, with the preserved area showing no division of muscle fibers and with clearly visible tissue organization (Fig. 3C). The cardiomyocyte nuclei (Fig. 3D), present 2 mm beyond the

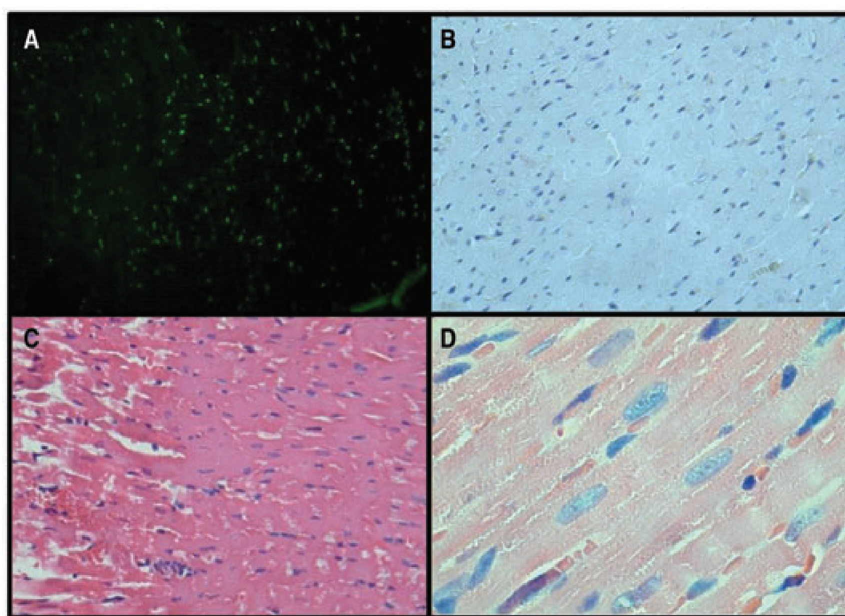

Figure 3. *Histological findings of radiofrequency lesions. (A) TUNEL (400× magnification) showing positive nuclear labeling with exposed DNA in region 2. (B) Anti-BAD immunohistochemistry (400× magnification). Note the absence of brown-stained nuclei, ruling out the presence of protein in this sample. (C) HE staining, transition zone located in region 2 (400× magnification). Note a visible delimitation of the ablated area. (D) Morphology of intact nuclei in region 2. No signs of apoptosis are seen; HE staining, 1,000×.*

transition area (region 2), were pycnotic, showing no evidence of membrane defragmentation, and there were no signs of cytoplasmic edema. These findings were quite consistent in all animals and no aspects suggestive of apoptosis were observed such as cellular involution, signs of chromatin condensation, or presence of apoptotic corpuscles.

TUNEL Assay

A total of 18 animals sacrificed 2 hours after ablation were used for this technique. Investigation of regions 1 and 2 of the tissue showed intense fluorescent labeling of the nuclei, indicating a positive reaction to the test suggesting the presence of apoptosis. Well-delimited fluorescent labeling of the nuclei occurred, corresponding to the ablated area, as observed by light microscopy of HE-stained slides (Fig. 3A). However, no labeling was observed in region 3, corresponding to the area free of apoptosis. These findings were quite consistent in all animals.

Immunohistochemistry

Immunohistochemistry was performed in the same 18 animals evaluated by TUNEL. The immunohistochemistry analyses with the anti-BAD and anti-caspase 3 antibodies were negative in all samples, as shown by the absence of brown staining in the cytoplasm, indicating nonexistent concentrations of intracellular proteins or insufficient concentrations for identification by the technique (Fig. 3B).

BAD—Western Blotting

Western Blotting was performed in 12 additional animals, three per time point studied (2, 24, 48, and 72 hours after ablation), which were used only for this procedure. The test was carried out without complications and with successful protein transfer to the membrane, as determined by Ponceau staining. No labeling corresponding to the molecular weight of the BAD protein (25 kD) was observed on the film after development, showing the absence of this protein in the tissues analyzed (Fig. 4).

Discussion

We have demonstrated for the first time that there is no induction of apoptosis at the periphery of RF lesions in the rat myocardium. Although the TUNEL test was clearly positive in this region, more specific evaluations such as immunohistochemistry, Western Blotting, and morphological criteria based on light microscopy were consistently negative for the presence of apoptosis in all animals studied.

Apoptosis is triggered by biochemical factors, one of the most important pathways including the release of cytochrome C after cell aggression.[7] The cytochrome C/Apaf-1 complex triggers the activation of caspases, proteins that cleave vital cytoplasmic and nuclear components resulting in DNA fragmentation. In addition, the family of BCL-2 proteins, known to be anti-apoptotic, includes a pro-apoptotic subclass known as BAD.[11] These adenosyl-triphosphate-dependent pro-apoptotic events include the activation of signaling factors that attract phagocytes without the activation of inflammatory mediators.[7] Apoptosis can be induced by different stimuli including hyperthermia,[10–14] which is mainly responsible for the formation of RF lesions.

However, the induction of apoptosis after *in situ* RF ablation has been little studied thus far. Our TUNEL assay showed positive results in region 2, corroborating the data reported by Winston et al.,[16] who investigated the periphery of the RF lesion in the myocardial tissue of pigs. Using the same technique, Rai et al.[15] assessed the cellular behavior of the hepatic tissue of pigs after ablation and also obtained positive results. The TUNEL assay is more sensitive than light microscopy and is routinely used to detect apoptosis *in situ*.[12] However, since TUNEL detects the terminal portion of damaged DNA, this method is of low specificity[17] and may detect nonspecific DNA damage. The TUNEL method aims to detect DNA strand breaks that occur during apoptosis. Nonetheless, necrosis—such as induced by RF—also produces DNA strand breaks and thus represents a target for TUNEL.[17] Accordingly, TUNEL assay may be useful for detecting ablated myocardium.[16] In contrast to the cited studies, in this investigation we set out to validate the TUNEL results by immunohistochemistry, Western Blotting, and morphological criteria based on microscopy, as currently recommended.[10,17]

In immunohistochemistry, we looked for the presence of central proteins involved in apoptosis (caspase and BAD) in tissue samples from the rat

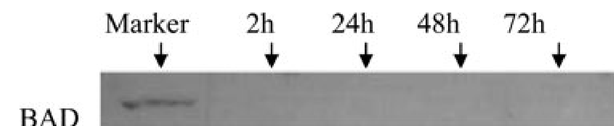

Figure 4. *Western Blotting membrane. Note on the left-hand side the presence of only the molecular weight marker (25 kD) with absence of the BAD protein in the subsequent lanes, represented here by animals sacrificed at 2, 24, 48, and 72 hours after ablation. This pattern was observed in all animals.*

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heart using anti-BAD and anti-caspase antibodies. The results did not reveal the presence of apoptosis in any of the regions studied (1, 2, or 3) in any animal. The analyses were repeated on different days with freshly prepared reagents and the positive control slide showed a reaction with well-defined results.

Since an apoptotic cell undergoes involution, its membrane does not rupture and there is no inflammation; we looked for these findings using microscopy. The histological analyses performed by two experienced pathologists detected no evidence of apoptosis in the nuclei of region 2. Structurally, the nuclei of this region are morphology compatible with the findings of region 1 and there are also nuclei of normal morphology, demonstrating that this region comprises the transition between the ablated area and the preserved area. However, Narula et al.[9] consider the screening of individual cardiomyocytes in apoptosis to be a difficult procedure.

Apoptosis typically starts rapidly after cell injury. In myocardial lesions caused by ischemia-reperfusion, apoptosis is detected after 2 hours.[13,14] The absence of BAD protein at all time points studied (2, 24, 48, and 72 hours after ablation) suggests that there was no late induction of apoptosis.

Interestingly, the two articles[15,16] that cited apoptosis after RF ablation used pigs, possibly suggesting that the TUNEL assay may not be precise for rats. However, various studies using ischemia/reperfusion and diabetes have validated the TUNEL assay in the species used in this study.[19,20]

Clinical Implications

Undesirable late effects may occur after RF ablation and seem to be related to late extension of the lesion beyond the area of acute coagulative necrosis.[2,3] The mechanisms responsible for extension of the lesion have not been clarified, but it has been postulated that the inflammatory response associated with the repair process resulting in fibrosis beyond the ablated area, damage to the microcirculation that surrounds the lesion, or ultrastructural damage beyond the area of coagulative necrosis may be involved.[3–6] Another possible mechanism of late extension of the lesion is apoptosis, which is a physiological cell process that counterbalances proliferation by mitosis. However, when apoptosis occurs in a deregulated manner it may result in tissue atrophy and organic dysfunction.[13,14] This study suggests that apoptosis is not induced at the periphery of the lesion. Thus, other mechanisms should be postulated and studied to clarify the late effects of RF.

Limitations

The study was conducted on the ventricular myocardium of healthy rats with no structural or electrical cardiac dysfunction, preventing direct extrapolation of the results to humans or to the AV node. We used 10-second ablations. Although improbable, it is possible that this time may not have been sufficiently long to induce apoptosis. The lesions monitored for more than 2 hours were evaluated only by Western Blotting and with a single protein for the identification of apoptosis (BAD). It is not possible to exclude the positivity of other pro-apoptotic proteins. The morphological criteria were assessed by light microscopy. We did not use electron microscopy, an important ultrastructural method for distinguishing between cell death by apoptosis and by necrosis.[10] However, the morphological findings were highly consistent and, in addition, the ultrastructural analysis of the periphery of the acute RF lesion (1 hour) did not reveal nuclei with apoptosis in the canine myocardium.[5] Finally, the presence of apoptosis would be unlikely to go unperceived by the various methods used in the study.

Conclusion

The present data suggest that RF ablation does not induce apoptosis in the ventricular myocardium of rats. This finding may have implications for the elucidation of late lesion extension following RF ablation.

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