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Apoptosis in kidney tissue is activated in an early period after radiofrequency ablation

Radijo dažnio abliacija ankstyvuoju poprocedūriniu laikotarpiu skatina inkstų audinio apoptozę

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Objective

Hyperthermia induced apoptosis may lead to tumor cell death thus expanding the volume of non-viable tissue and warrant a "safety margin" of at least 10mm to exclude the possibility of tumor recurrence. We carried out an experimental study to investigate the cellular injury produced by radiofrequency ablation in the area surrounding the ablated tissue and to describe early apoptotic processes in the transition zone following radiofrequency ablation procedure in a porcine kidney model.

Materials and methods

Eight anesthetized pigs underwent laparotomy and local thermal ablation of the kidney parenchyma. The ablated tissue and the surrounding parenchyma were investigated for apoptosis applying Western blot analysis and immunohistochemistry.

Results

The active (cleaved) caspase-3 17-kDa subunit was detected in the transition zone one hour after ablative procedure at a distance of 7-9 mm from the rim of the necrosis zone. In contrast analysis of tissues in necrosis zone and in surrounding normal kidney parenchyma revealed no markers of apoptotic activity.

Conclusions

We determined that apoptosis, leading to further cell death, is activated in the majority of cells in the transition zone, thus supporting the hypothesis that the "safety margin" of 8 mm is encompassed by the indirect thermal effect.

Key words: kidney cancer, radiofrequency ablation, apoptosis, caspase-3

Įvadas
Hipertermijos indukuojama apoptozė gali lemti vėžinių ląstelių žūtį ir taip praplėsti radijo dažnio abliacijos saugią gydymo ribą net iki 10 milimetrų, taip užkertant kelią ligai atsinaujinti. Hipotezei patvirtinti atlikome eksperimentinį tyrimą, kuriuo siekėme įvertinti radijo dažnio abliacijos poveikį inkstų ląstelėms audinyje aplink susidariusią nekrozės zoną bei apibūdinti ankstyvus apoptozinius procesus šioje zonoje.

Metodai

Sukėlus bendrąją endotrachėjinę nejautrą, operuotos aštuonios eksperimentinės kiaulės. Joms atliktos vidurinės laparotomijos ir inkstų parenchimos radijo dažnio abliacija. Abliacijos zona ir ją supantys audiniai buvo tiriami Western bloto bei imunohistochemijos metodais siekiant nustatyti apoptozę.

Rezultatai

Valandą po procedūros aktyvuota trečiosios kaspazės 17-kDa dalis nustatyta tranzitorinėje abliacijos zonoje 7–9 mm atstumu nuo nekrozės ribos. Tiriant nekrozės zoną bei aplink nepažeistą inksto parenchimą, apoptozės žymenų aktyvumas nebuvo nustatytas.

Išvados

Mes nustatėme, kad po radijo dažnio abliacijos aštuonių milimetrų atstumu nuo nekrozės zonos daugumoje inksto ląstelių yra aktyvuojama ląstelių žūtį lemianti apoptozė. Tai patvirtina hipotezę, kad po šios procedūros dėl netiesiogiai plintančio terminio poveikio aplink nekrozės zoną gali susidaryti papildomai saugi 8 mm zona.

Reikšminiai žodžiai: inkstų vėžys, radijo dažnio abliacija, apoptozė, kaspazė-3

Introduction

The radical nephrectomy has been the "gold standard" for the treatment of clinically localized renal cell carcinoma, but nephron sparing techniques have shown that oncologic and functional outcomes are equivalent to those with radical nephrectomy for patients with renal tumors 4 cm or smaller in size [1–3].

More recently, several new minimally invasive nephron sparing techniques such as renal cryotherapy, high-intensity focused ultrasonography, laser ablation, microwave ablation and radiofrequency ablation (RFA), have been used to treat small renal tumors in selected patients [4].

These techniques offer advantages over extirpative techniques by reducing perioperative morbidity, shortening the hospital stay, promoting faster recovery, and importantly, potentially treating patients who are poor surgical candidates while preserving renal parenchyma and function [5, 6]. RFA use has been described in patients with small renal tumors (<4cm) who have poor renal reserve, multiple bilateral RCC in Von Hippel Lindau syndrome, or hereditary renal cell carcinoma's. Recently, RFA was aggressive enough, because the ablation covered the tumor and minimum 5–10 mm of surrounding normal parenchyma ("safety margin") to exclude the possibility of tumor recurrence. As a consequence of this, the rate of complications increased when bigger tumors were ablated. The main complications of this procedure are the thermal injuries of surrounding organs and tissues such as ureters, surrounding bowel, genitofemoral and ilioinguinal nerves, psoas muscle, adrenal gland and diaphragm. Retroperitoneal hematomas and damage of renal collective system also can occur [7].

RFA works by converting radiofrequency waves into heat, resulting in thermal damage to parenchymal tissue [8]. Four zones of cellular changes are described in ablated tissue following treatment: application, central, transition, and reference tissue zones. The application zone is proximate to the heat source. The central zone immediately surrounds the application zone and consists of necrotic tissue. The transition zone contains apparently undamaged tissue but exhibits signs of subacute hemorrhage. The reference zone refers to normal tissue surrounding the transition zone [9].

It is well established that temperatures above 50 °C results in immediate cell death and prolonged hyperthermia above 40 °C which is registered in tumor surrounding parenchyma (transitional zone) already leads to inactivation of vital enzymes and may initiate apoptosis which will lead to further cell death [10, 11].

Clinical dilemma today – if it is possible to bring the ablation margin as close as possible to the tumor rim and do not compromise treatment efficacy and consequently reduce complication rate. Therefore we hypothesized

that the volume of non-viable tissue may expand because of hyperthermia induced apoptosis in transitional zone. Thus the characterization of on-going apoptosis in transitional zone cells has clinical significance. The aim of our study was to describe apoptotic processes in the transition zone following RFA procedure in a porcine kidney model.

Materials and methods

Approval of the study protocol was obtained from the Ethics Committee at the State Food and Veterinary Service of Lithuania. White land-race pigs were chosen as the study species. The eight pigs weighting from 25 to 35 kg underwent RFA procedure under general endotracheal anesthesia.

Anesthesia

For the premedication the i.m. injection of Fentanyl 2 μg/kg and Midazolam 2 mg/kg was used. Afterwards the animals were laid supine on the operating table. Anesthesia was induced with Propofol 8 mg/kg. After intubation the anesthesia and analgesia was maintained with 2% Izoflurane and oxygen compound, and Fentanyl 10 μg/kg/h. The arterial blood pressure was measured directly via femoral artery catheterization. During surgery intravascular volume was maintained using normal saline infusion 10 ml/kg/h. The parameters of oxygen saturation, arterial blood pressure, central venous pressure, body temperature, and heart electric activity (electrocardiogram) were monitored continuously.

Operation

After anesthesia the midline laparotomy was performed and both kidneys were mobilized. The radiofrequency ablation was carried out using ELEKTROTOM HiTT 106 (BERCHTOLD Medizinelektronik Tuttlingen, Germany) radiofrequency generator. An internally cooled monopolar electrode with 2 cm exposed tip length and power output of 25 W were used to ablate tissue in the left and the right kidney. RFA was carried out for 10 minutes. Tissue temperatures were acquired by type K thermocouple during the procedure at 5 mm and 10 mm distance from the active electrode. One hour after the experiment all the animals were sacrificed by injecting Pentobarbital 100 mg/kg intravenously. The death was confirmed by isoline on electrocardiogram for 5 min. Consequently, both kidneys were explanted and the ablated zones with surrounding normal kidney parenchyma were excised. Sixteen consistent lesions were used for further analysis. Each specimen was divided longitudinally in two equal parts. Three pieces of tissue approximately 4 mm^3 in volume were cut out from the central zone, transition zone, and the undamaged kidney parenchyma of the first part of the specimen. The biopsies were snapping frozen in liquid nitrogen. Frozen specimens weighed 300 μg approximately.

The section of 3–4 mm in thickness was sliced in parallel with the division surface from the second part of the specimen. Each section contained application, central, and transition zones surrounded by undamaged kidney tissue. These tissue slices were fixed in 10% Formaldehyde and embedded in paraffin. Subsequently the 4 μm sections of the paraffin blocks were made, deparaffinised and stained with hematoxylin and eosin for histological assessment.

Western blot analysis

The frozen tissue samples were crushed in RIPA buffer (Tris-HCl 50 mM, pH-6.8, NaCl 150 mM, 0.1% SDS, 1% NP-40, 0.5% Na-deoxycholate) with 500 mM PMSF and the inhibitor of the proteases. The extracts were centrifuged 5 minutes at 14,000 rpm in 4 °C temperature. The concentration of superficial fraction of proteins was measured by spectrophotometry. Equal amounts of protein extracts were fractionated on a 10% Bis Tris gel (Bio-Rad Laboratories; Hercules, CA). After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. Ponceau S (Sigma; Deisenhofen, Germany) staining of the membrane was used to ensure equal loading. For immunoblotting, unspecific binding was blocked with 5% non-fat milk, and the membranes were then incubated with the primary rabbit polyclonal anti-caspase-3 antibody (Sigma; Deisenhofen, Germany) in a dilution of 1:250 in 5% non-fat milk TBS-Tween at 4 °C overnight. Subsequently, the membranes were washed with TBS-Tween buffer (5% non-fat milk in 20 mM Tris-HCL, 150 mM NaCl, 0.1% Tween 20) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad

Laboratories; Hercules, CA) at room temperature (RT) for 1 h. After washing with TBS-Tween buffer, the color development was performed with color reagents of Bio-Rad (Bio-Rad Laboratories; Hercules, CA).

Immunohistochemistry

Paraffin sections (4–5 μm) were deparaffinised and rehydrated using the standard xylene and ethanol immersion technique**.** Antigen retrieval was done by rinsing tissue sections with 1 μg/ml proteinase K (Sigma # P-4914) for 10 minutes at room-temperature. Sections were incubated for 30 min with a rabbit anti-human caspase-3 (Sigma#C8487) antibody, diluted 1:400 in 1% BSA in TBS. For negative control slides were incubated in 1% BSA in TBS. After rinsing 2 times in TBS for 5 min, sections were incubated for 30 minutes at room temperature with Dako REAL™ EnVision™/HRP goat anti-rabbit/mouse antibodies (DAKO; Hamburg, Germany), rinsed 2 times in TBS, further processed using 20 μL the DAB-containing Substrate Working Solution (CHROM) and 1 mL Dako REAL™ Substrate Buffer complex. The slides were counterstained with aqueous haematoxylin, rinsed well in water and dehydrated through alcohols and xylene and cover slipped for viewing. Visualization was performed by scanning the slides with confocal microscopy (Leica GmbH; Wetzlar, Germany).

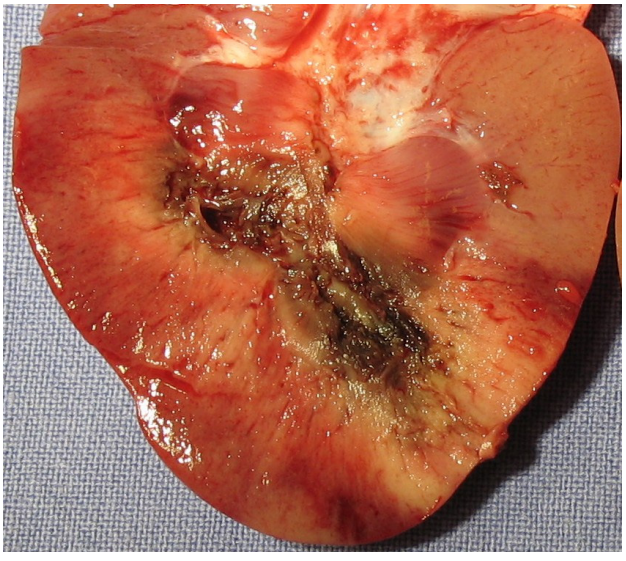

Figure 1. Macroscopically the ablation site was ellipsoid shaped with the pale kidney tissue in the central part (necrosis zone) surrounded by the hyperemic rim (transitional zone)

Results

Macroscopically the ablation site was ellipsoid shaped with the pale kidney tissue in the central part surrounded by the hyperemic rim (Fig. 1). The diameter of longitudinal dimension of the whole affected area was 30±3 mm and the transverse dimension was 20±2 mm. The diameters of the necrosis zone were 22±1 mm and 12±1 mm respectively. Morphologic analysis of the sections of the affected area stained with hematoxylin and eosin revealed two zones: the inner zone of tissue necrosis (central zone) and transition zone. The necrotic tissue with damaged structure of kidney cells was observed in the central zone. The transition zone con-

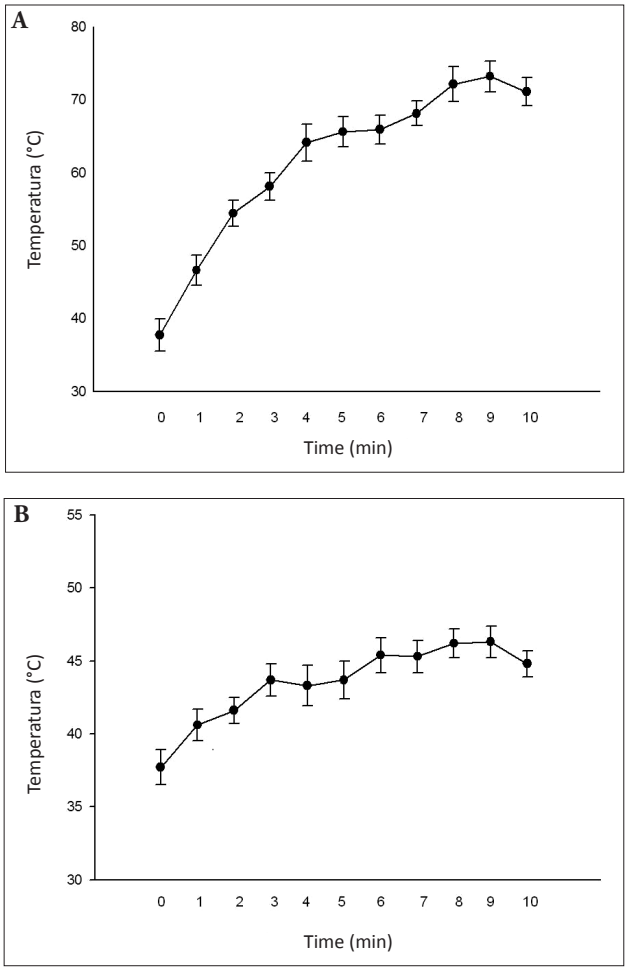

Figure 2. A. Temperatures above 70°C were registered at the distance of 5 mm from the active electrode (central zone) during RFA procedure; B. At the distance of 10 mm from the active electrode the highest temperatures ranged from 42 to 46 °C

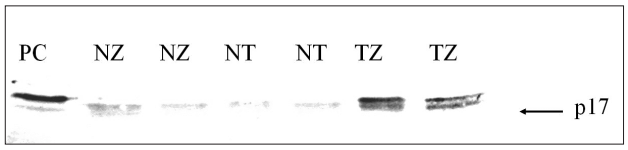

Figure 3. The active 17-kDa subunit of caspase-3 was detected by Western blot analysis in the transition zone of the ablated tissue in contrast to normal kidney parenchyma. PC – positive control; NZ – necrotic zone; NT – normal tissue; TZ – transitional zone

tained apparently undamaged kidney cells with signs of sub-acute hemorrhage manifesting as tissue infiltration with blood cells. Cells in this zone were eosinophilic and showed condensed chromatin, however intercellular connections were maintained. The tissue adjacent to the transition zone was morphologically unchanged. The temperatures at the distance of 5mm from the active electrode (central zone) peaked to 72.0 °C (Fig. 2A). It increased for 8 minutes to reach an average temperature of 70°C and stabilized thereafter. At the distance of 10mm from the active electrode the temperatures increased up to 46,4°C (Fig. 2B). Western blot analysis detected the active 17-kDa subunit of caspase-3 in the transition zone of the ablated tissue one hour after RFA. There was no expression of the active 17-kDa subunit of caspase-3 in the normal kidney tissue surrounding the ablation area (Fig. 3).

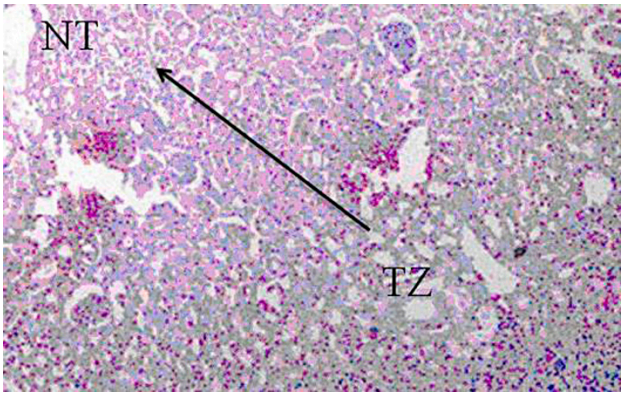

Figure 4. There was an intense immunoreactivity of active (cleaved) subunit of caspase-3 in kidney cells within transition zone (TZ), whereas no immunoreactivity in normal kidney parenchyma (NT) was present. (Immunohistochemical stain; original magnification, ×20)

In parallel immunohistochemistry revealed an intense immunoreactivity of active (cleaved) subunit of caspase-3 demonstrating the ongoing process of apoptosis in kidney cells within transition zone, whereas no immunoreactivity was present in normal kidney parenchyma (Fig. 4). Moreover, the immunoreactivity of active caspase-3 was present in vast majority of kidney cells within transition zone at a distance of more than 8 mm from the rim of the central (necrosis) zone.

Discussion

In this experiment we demonstrated the biological effect of RFA on induction of apoptosis in the transitional zone in kidney parenchyma. A better understanding of the biological processes in RFA kidney may lead us to improve radiofrequency technique in kidney tumors. This could include the reduction of the ablated area, decrease number of electrodes required for adequate ablation. Therefore this could increase a wider preservation of kidney parenchyma and lower the amount of complications associated to the treatment.

Focal hyperthermia induces tumor destruction by both direct and indirect thermal effects. The direct thermal effect reflects local increase in temperature resulting in immediate cell death. Direct heat injury is followed secondary indirect injury. Clinical and experimental data indicate that tissue injury progresses after the cessation of the focal hyperthermic stimulus. The exact mechanism of this process is undetermined. The full extent of tissue damage becomes evident 1 to 7 days after focal hyperthermia application. One of the factors in progression of injury is apoptosis [12].

Apoptosis is a distinctive form of cell death manifested by characteristic chromatin condensation and DNA fragmentation [13]. It is well established that apoptosis increases in a temperature-dependent manner. Temperatures between 40 °C and 45 °C cause inactivation of vital enzymes and may initiate apoptosis [10, 11]. Recently, new methods for the detection of specific parts of the apoptotic pathway became available such as the detection of caspase activity, specifically caspase-3, which is essential for DNA fragmentation [13, 14].

In our study the hyperthermic temperature, which could induce apoptosis was registered at 10mm from the active probe and this coincided with the transitional

zone. Moreover, we detected an active caspase-3 subunit in transitional zone using two different techniques. The determination of apoptosis in transitional zone is clinically important because all radiological examinations demonstrates only necrotic zone as non-viable tissues but surrounding transitional zone is said to be viable but ongoing apoptosis in this zone will lead to further cell death and the expansion of necrosis.

Zlotta *et al* in their study found that more extensive necrosis was observed in the radiofrequency induced lesion of the renal specimen removed 1 week after RFA compared to specimens obtained immediately after RFA [15]. There are some studies made which describe apoptotic processes in transitional zone after RFA in liver parenchyma [14, 16]. To our knowledge, our study is the first report in which the ongoing apoptosis in transitional zone of kidney parenchyma was described after RFA. Our study findings replicates to data published by Vanagas et al. where they reported apoptotic activity in transitional zone in porcine liver RFA model [9].

In conclusion we determined that apoptosis, leading to further cell death, is activated in the majority of cells at a distance of more than 8mm from the rim of necrosis zone. This finding supports the hypothesis that the "safety margin" of 8 mm is encompassed by the indirect progressive thermal effect of hyperthermia during RFA procedure.

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