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# Tackling hypertension via adrenal ablation - the development of novel immunohistochemical ablation biomarkers

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**Abstract—** Microwave thermal ablation is under consideration for minimally invasive treatment of unilateral and bilateral adrenal adenomas, symptomatic of Conn's syndrome. Currently available microwave technologies are ill-suited to precise ablation of small adrenal targets. In this study, we report on the use of immunohistochemical markers as accurate microscopic markers of ablation damage extent. This ex vivo study will aid in efforts towards the design of microwave ablation systems for targeting adrenal masses. Microwave ablation was carried out on ex vivo bovine adrenal glands using various power outputs and ablation durations. The area of damage was assessed using histopathological, immunohistochemical and densitometry techniques. The immunohistochemical analysis illustrated the feasibility of this technique to microscopically detect the area of cell damage in tissue accurately. These results will aid in further investigation and development of microwave technology for precise ablation of adrenal masses.

**Index Terms—**microwave ablation, histology, adrenal gland.

## I. INTRODUCTION

Primary aldosteronism (PA) is the commonest secondary cause of hypertension, accounting for 6-20% of all cases [1,2,3]. It is characterized by excessive aldosterone production by unilateral or bilateral aldosterone producing adenomas (APA). Current definitive surgical management of APAs removes the entire adrenal gland and therefore is unsuitable for the treatment of bilateral disease (circa 60% of all PA) due to inevitable adrenocortical insufficiency. Consequently, there is a need for optimised, minimally invasive therapies that definitively treat PA while simultaneously preserving normal adrenocortical function. Thermal ablation provides a plausible, minimally invasive technology to approach definitive management of PA [4].

Radiofrequency ablation (RFA) has been used to manage adrenal adenomas in small observational clinical studies [5]. However, while therapeutically efficacious, RFA did not achieve the necessary precision ablation to selectively target adrenocortical adenomas, while preserving adjacent normal adrenal cortex. Moreover, indiscriminate ablation of adjacent adrenal medulla using

techniques of lower precision can potentially precipitate catecholamine crisis. [6] Microwave ablation (MWA) may represent a modality which can achieve this precision and potentially offer a definitive therapeutic option for unilateral and bilaterally driven PA.

During a microwave ablation (MWA) procedure, the antenna (typically enclosed in a needle or rigid catheter) is introduced into the target under ultrasound or Computed Tomography (CT) image guidance. Microwave power radiated by the antenna is absorbed by surrounding tissue, which leads to cell death due to elevated temperatures, characterised microscopically by coagulative necrosis. [7] Many clinical studies have been reported on use of MWA for treatment of tumors in the liver, lung, kidney, and breast [8]. Current MWA systems in clinical use operate at 915 or 2450 MHz. The latter frequency provides shorter antenna lengths, and more rapid heating [9]. Existing MWA systems have largely been optimized for creating large ablation zones, as is required for treating tumors in vascular organs such as the liver. Due to the small size of adrenal glands and adenomas, devices that afford creation of spherical ablation zones up to 10 mm in diameter are required. In previous studies, computational and experimental studies showed that microwave technologies can produce such ablation zones [4].

Macroscopic and light microscopic techniques are normally used to assess the extent of ablation zones following application of MWA. The latter typically assesses the presence of coagulative necrosis and requires that experiments are undertaken on in vivo tissue only. However, modelling of MWA technology is often carried out on ex vivo cadaveric tissue. We demonstrate that immunohistochemical evaluation of damage associated molecular patterns (DAMPs), provides a precision methodology for assessing the extent of ablation zones in ex vivo. These molecules are released extracellularly only following cell injury. [10]. This study reports on expression of two DAMP molecules, namely heat shock protein 70 (HSP-70) and high mobility group box 1 (HMGB-1) following the use of microwave ablation on ex vivo bovine adrenal tissue at 2.45 GHz using a lateral

approach. Section II details the ablation of adrenal glands, pathological and immunohistochemical (IHC) analysis. Results from the pathology and IHC analysis are provided in Section III. In Section IV, results are analysed and discussed.

## II. MATERIALS AND METHODS

### A. Microwave thermal ablation of ex-vivo adrenal glands

Bovine adrenal glands were obtained from a local abattoir, in the first 4 hours post mortem and transported directly to the laboratory. The design and validation of the microwave antenna has been presented in [4]. We used a microwave generator (Sairem, 2.45GHz, Neyron, France) to power the antenna. The antenna was placed laterally between the surface of the gland and the perirenal/perinephric fat, providing an insulating layer and mimicking in vivo conditions. Three power and time settings were used as follows: frequencies at 75W for 50 seconds (s), 50W for 50s, and 50W for 30s.



Fig. 1 Microwave probe and antenna design used for ablation.

### B. Pathological analysis

Immediately following ablation each adrenal gland was formalin fixed overnight using a tissue processor (Lecia, ASP300S,IL,US) and embedded in paraffin wax the following morning. Slides were generated following tissue sectioning (5  $\mu$ m). The tissues were stained with haematoxylin and eosin (H&E) for basic histopathological assessment.

### C. Immunohistochemical staining

Mouse anti-human monoclonal antibodies, with cross-reactivity for bovine adrenal and specific for HSP-70 and HMGB-1 (Abcam, Cambridge, UK) were used to identify DAMP expression. The tissue samples were stained using the following procedure: 5  $\mu$ m sections were placed on glass slides and heat mediated antigen retrieval was performed using citrate buffer in a pressure cooker. Endogenous peroxidases were neutralised by incubating the tissue slides in a hydrogen peroxide / methanol solution for 30 mins. Nonspecific binding was prevented by incubating the tissue in 2.5% horse serum for two hours. The slides were incubated in primary antibody overnight at 4°C and afterwards incubated with secondary antibody for 1.5 hours. Finally, slides were developed using diaminobenzidine (DAB) and a coverslip applied for imaging.

### D. Microscopy and analysis

The stained slides were scanned using a virtual slide scanner (Olympus, VS120, Tokyo, Japan). This scanner generated virtual slide images (VSIs) which can be viewed

using the associated Olyvia® (Ver. 2.9, Olympus, Tokyo, Japan) software. Overview images of the tissues were taken at 2X magnification, while images at 40X were taken at 1mm increments into the tissue from the probe contact area. The 40X images were analysed using densitometry software on ImageJ® (Ver. 1.51, NIH, MD, US), to assess the percentage of DAB staining and intensity. The densitometry of the outer cortex and inner medulla of the adrenal gland were assessed separately as their broadband dielectric properties are significantly different. The difference in dielectric properties between the two tissues mean they react very differently in response to MWA.

### E. Statistical Analysis

The Graphpad Prism 7® software package was used for statistical analysis. The immunohistochemistry results were compared using a chi-squared test. A p-value of less than 0.05 was considered significant.

## III. RESULTS

### A. Histopathological Analysis

Fig 2. demonstrates H&E staining of bovine adrenal gland post MWA. Areas, identified macroscopically as damaged tissue, appear more eosinophilic (dark purple), than unaffected adjacent tissue. However, no obvious characteristics of coagulative necrosis e.g. nuclear decay were visible in any of the three ablated tissue samples. [10]

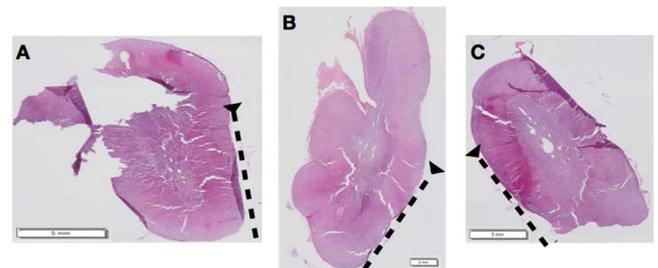


Fig. 2 H&E stained tissues post ablation: (A) 50W for 30s, (B) 50W for 50s, and (C) 75W for 50s. Dotted line represents MWA probe placement during ablation procedure.

### B. HMGB1 Immunohistochemical Analysis

Fig. 3, A(i), B(i) and C(i) demonstrate immunohistochemical staining for HMGB1 expression on adrenal tissue post-ablation. Supporting densitometry is demonstrated in Fig 4. No HMGB-1 expression was seen at 50W for 30s (Fig. 3Ai & Fig. 4A), with no significant difference between the area adjacent to probe insertion versus the internal control i.e. the tissue area most distant from the probe. Low level HMGB1 expression was demonstrated at 50W for 50s (Fig 3Bi & Fig. 4B), with stronger expression at 75W for 50s.

Hence, a clear dose-dependent relationship was seen between MWA application and HMGB1 expression. Expression of HMGB1 in adrenal medullary tissue was consistently higher than that of adrenal cortex, reflecting the difference in dielectric properties between the medulla and cortex [4]. HMGB1 expression in both the cortex and

medulla was highest adjacent to the probe contact area and significantly dropped in expression with each 1mm increment away from the probe contact area (Fig 3 & 4).

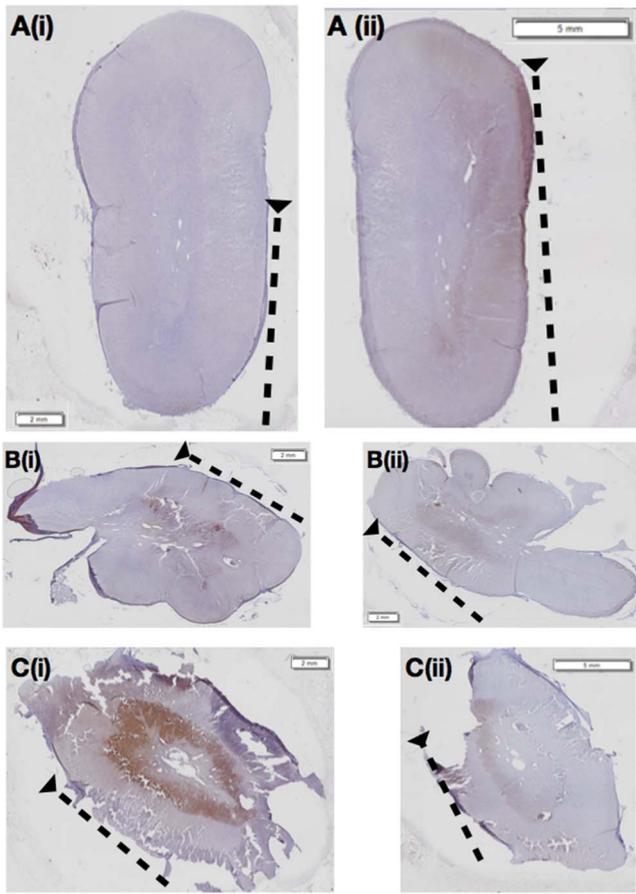


Fig 3. HMGB1 staining of tissue post ablation: (A) 50W for 30s, (B) 50W for 50s, and (C) 75W for 50s, (i) HMGB1, (ii) HSP-70

### C. HSP-70 Immunohistochemical Staining

Fig 3. A(ii), B(ii), C(ii) demonstrate immunohistochemical staining for HSP-70 expression on adrenal tissue post-ablation. Supporting densitometry is demonstrated in Fig 5. HSP-70 expression was detected at all MWA power and time settings. The levels of HSP-70 expression do not increase in correlation with the power and time settings used. It can be seen that the medullary tissue exhibited higher HSP-70 expression in comparison to the cortical tissue reflective of their dielectric properties. Densitometry found that HSP-70 expression in both the cortex and medulla was highest adjacent to the probe contact area and significantly dropped in expression with each 1mm increment away from the probe contact area. (Fig 3 & 5)

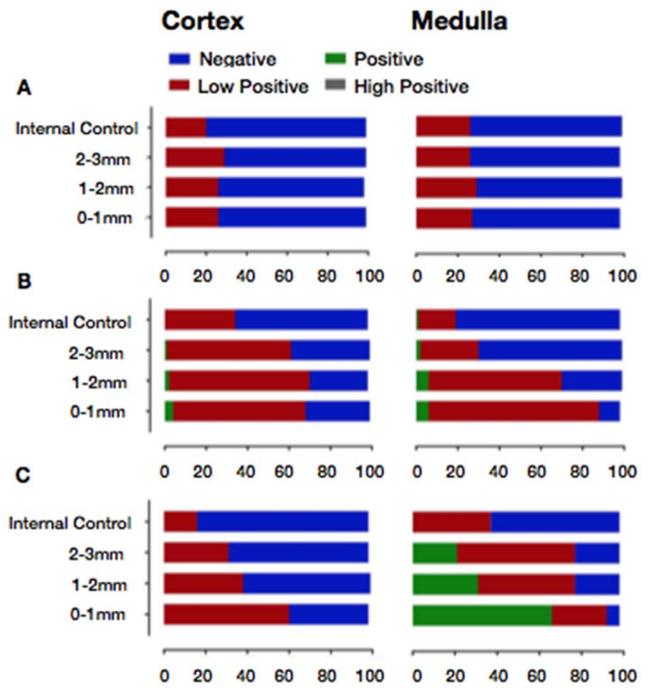


Fig 4. HMGB1 densitometry: (A) 50W for 30s, (B) 50W for 50s, and (C) 75W for 50s

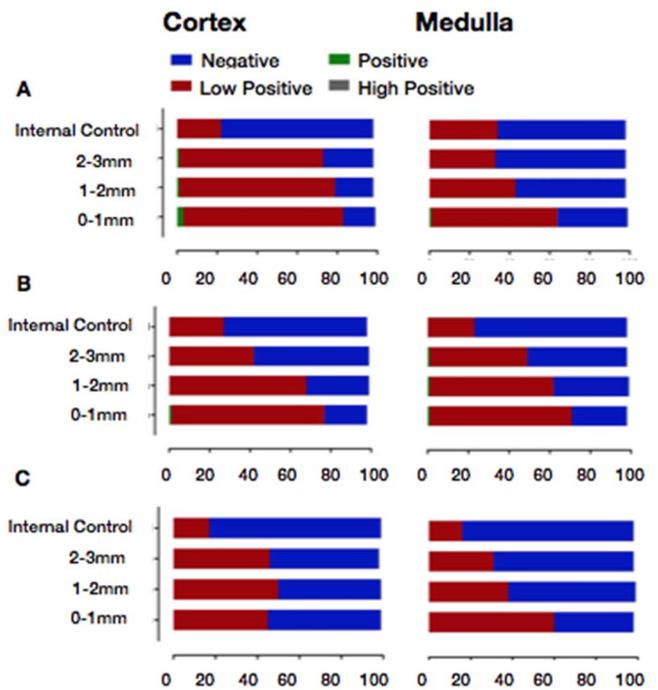


Fig 5. HSP-70 densitometry: (A) 50W for 30s, (B) 50W for 50s, and (C) 75W for 50s

#### D. Depth of Damage

HSP-70 staining was also used to assess the depth of adrenal tissue damage from the probe tip, given that this DAMP marker was present at detected at lower power and time settings. Moreover, HSP-70 demonstrated more uniform and accurate staining patterns compared with HMGB1. The extent of HSP-70 staining reduced at increasing tissue depths distal to the area of probe contact.

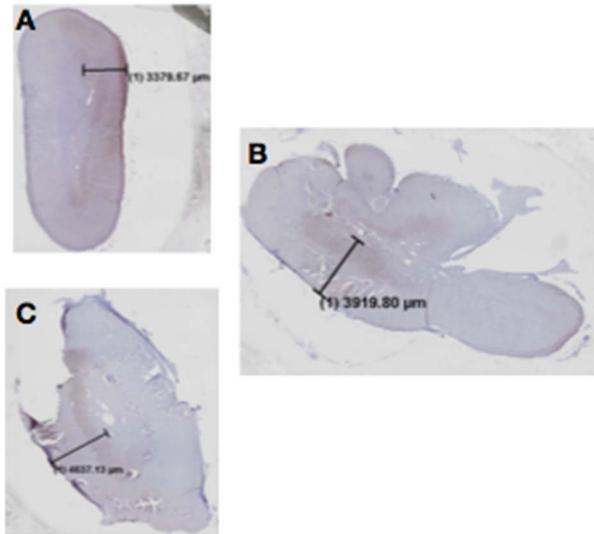


Fig 6. Depth of damage assessed using HSP-70: (A) 50W for 30s; 3.3mm, (B) 50W for 50s; 3.9mm, and (C) 75W for 50s; 4.6mm

#### IV. DISCUSSION AND CONCLUSIONS

This study demonstrates expression of the DAMPs, HSP-70 and HMGB1 in response to MWA of ex vivo bovine adrenal tissue. The expression of both molecules provides useful information regarding the pattern and extent of ablation achieved at varying applied doses of MWA. In this regard, HMGB1 exhibited a dose-dependent pattern, with greater expression evident at higher voltages and duration of radiation exposure. HSP-70 provided an early indicator of damage which clearly demonstrated the pattern and depth of ablation from probe tip outwards. As expected, the extent of adrenocortical damage was lower in tissue more distal from the applicator probe tip. In line with higher dielectric properties, the adrenal medulla exhibited higher sensitivity to MWA when compared to adrenal cortex.

Several important factors are highlighted for consideration in relation to MWA in adrenal tissue. Firstly, immunohistochemical evaluation of DAMPs can provide a useful and precision microscopic measurement for tissue damage during ex vivo adrenal modelling of MWA. Importantly, the relative extent of medullary and cortical damage can be accurately assessed using this methodology. Notably a clear differential in tissue sensitivity was ascertained whereby, medulla demonstrated higher sensitivity to tissue damage when compared to cortex.

Overall, this study evaluated a tissue sparing approach to adrenal ablation using limited application of MWA in an effort to preserve adrenocortical tissue adjacent to the

ablation zone, and to avoid medullary damage. We demonstrated using DAMP expression, that adrenocortical tissue-sparing application of MWA presents a feasible option going forward. However, application of MWA, sufficient to cause adrenocortical damage induced significantly greater medullary damage. This informs our future approach to in vivo studies going forward whereby, pre-medication with adrenoreceptor antagonists will be necessary to avoid potential catecholamine crisis even during limited adrenal ablation procedures.

Limitations of this study include the absence of an adrenocortical tumour model and the ex vivo nature of investigation. In future studies, these limitations will be addressed using animal models of functioning adrenocortical tumours and adrenocortical sufficiency post-ablation. Moreover, we will validate our ablation findings in ex vivo tissue against an in vivo model using DAMP expression.

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