Abstract

INTRODUCTION: Vessel wall MRI (vwMRI) can improve evaluation of intracranial atherosclerotic disease (ICAD). However, pathological validation is needed to improve vwMRI techniques. Human pathology samples are not practical for such analysis, so an animal model is therefore needed.

MATERIALS AND METHODS: Watanabe heritable hyperlipidemic (WHHL) rabbits and apolipoprotein E (ApoE) knockout rabbits were evaluated against New Zealand white (NZW) wild type rabbits. Evaluation of intracranial arteries was performed with vwMRI and pathological analysis, rating the presence and severity of disease in each segment. Two-tailed t-tests were performed to compare disease occurrence and severity prevalence among rabbit subtypes. Sensitivity and specificity were calculated to assess diagnostic accuracy of vwMRI.

RESULTS: 17 rabbits (5 WHHL, 4 ApoE knockout, and 8 NZW) were analyzed for a total of 51 artery segments. 11 segments (5 WHHL, 6 ApoE knockout) demonstrated ICAD on pathology. Disease model animals had lesions more frequently than NZW animals ($p<0.001$). Sensitivity and specificity of vwMRI for detection of ICAD were 68.889.5% and 95.21.9%, respectively. Excluding mild cases to assess vwMRI accuracy for detecting moderate to severe ICAD lesions, sensitivity improved to 100% with unchanged specificity.

CONCLUSION: ICAD can be reliably produced and detected using 3T vwMRI-compatible WHHL and ApoE rabbit models. Further analysis is needed to better characterize development and progression of the disease to correlate tissue-validated animal findings with those on human vwMRI studies.

Introduction

Ischemic stroke is a leading cause of death and long-term disability. Intracranial atherosclerotic disease (ICAD) causes 15% of ischemic strokes in the United States and up to 50% in some populations, representing the most common etiology of ischemic stroke worldwide.(1–4) ICAD pathophysiology and natural history are incompletely understood. Resultantly, there is significant variability in diagnostic and treatment approaches. Identifying the optimal treatment for the large number of individuals with ICAD therefore remains a difficult problem.

Vessel wall magnetic resonance imaging (vwMRI) techniques offer new information for the management of ICAD.(5–9) In general, vwMRI improves characterization of vascular disorders by allowing direct visualization of vessel wall pathology as opposed to inferring minimal information from standard luminal imaging techniques. While promising as a diagnostic and prognostic tool for ICAD evaluation, vwMRI currently lacks support from histopathological confirmation, the gold standard in the assessment of human disease. This paucity of tissue data is true for nearly all ICAD investigation and is a particular problem when considering vwMRI investigations of ICAD. Such pathology studies are essentially impossible to perform in humans for a number of reasons, including the prohibitive morbidity of biopsy and typical timing of diagnosis late in the course of the disease.

In settings such as this, in which a disease is difficult to adequately characterize in humans, animal models must be used to elucidate critical natural history and pathophysiological data. The barrier to progress is the lack of a vwMRI-compatible animal model for preclinical studies and, in particular, one using 3T magnet strength and sequences that can be easily transitioned to human studies and clinical practice. One potential candidate is the Watanabe

heritable hyperlipidemic (WHHL) rabbit model, which is thus far the most extensively utilized atherosclerotic animal known to develop ICAD.(10–13) Despite being the most commonly studied, the mechanism by which it develops ICAD does not replicate the way by which ICAD develops in the vast majority of humans.(11–15) Furthermore, supply of these animals has recently proven unreliable, so the need for a more viable model is acute.(16) Diet-based models exist for atherosclerosis, such as producing disease in wild type New Zealand white (NZW) rabbits fed high cholesterol diets. However, systemic effects typically lead to animal demise before ICAD occurs.(17) Additional rabbit models of extracranial atherosclerosis exist, most notably using apolipoprotein E (ApoE) knockout rabbits, yet investigation for development of intracranial atherosclerosis is lacking to date.(13,18,19). This study seeks to demonstrate the ability to effectively perform vwMRI in rabbits and assess suitability of the ApoE knockout rabbit as a potential alternative to the WHHL model ICAD analysis, using histopathological correlation that is unavailable in human studies.

Methods

All animal investigation was performed according to a protocol approved by the institutional animal care and use committee at our academic medical center. All rabbits ~~at~~ underwent serial vwMRI studies on a 3T Prisma scanner (Siemens Healthineers, Erlangen, Germany) using an ankle coil over the head to obtain pre- and post-contrast DANTE T1 SPACE, T2 SPACE, and TOF MRA.(17) Table 1 summarizes acquisition parameters. For gadolinium contrast, Multihance (gadobenate dimeglumine, Bracco, Milan, Italy) dose was calculated by animal mass (0.2ml/kg, 0.1 mmol/kg).

Initial investigation was performed on mature WHHL rabbits. During the course of the study, additional WHHL rabbits became unavailable due to logistical limitations at the breeding facility.(16) Mature ApoE knockout rabbits fed a custom 2% cholesterol diet (Envigo Teklad Diets, Madison, WI) underwent the same imaging protocol. Additionally, wild-type NZW rabbits were evaluated, according to the same protocol, to serve as normal controls.

vwMRI images obtained closest to euthanasia were analyzed by a board-certified radiologist with certificate of added qualification in neuroradiology. Basilar and internal carotid artery segments were each rated as having no, mild, moderate, or advanced ICAD on vwMRI. Grading was based on subjective assessment of plaque volume, signal abnormality, stenosis, and enhancement.

When determined appropriate to evaluate a certain stage of disease or dictated by failure to thrive, euthanasia was performed with perfusion fixation following a standardized protocol. General endotracheal anesthesia was initiated and maintained using isoflurane. The hair over the neck was shaved and a midline incision over the trachea made with a scalpel. Using blunt dissection, the right carotid artery was isolated. Isolating the artery under gentle tension with vessel loops, the artery was entered with a 22 gauge intravenous catheter. The catheter was gently advanced over the needle, which was used for forming an arteriotomy. The needle was removed and a 0.018 inch wire advanced into the catheter. The catheter was removed over the wire and a 5 French micropuncture catheter advanced over the wire. The catheter was secured by suture over the vessel segment with the indwelling catheter. The inner catheter and wire were removed, and perfusion was performed by delivering a steady flow of 2% paraformaldehyde and 5% glutaraldehyde solution with a perfusion pump. With perfusate flowing, a jugular vein was transected to exsanguinate the animal with the aim of achieving fixation with few blood cells in

vessels. The animal was decapitated and the brain, with intact intracranial arteries, harvested and placed in a container with formalin.

Pathological preparation and analysis was performed by a specialized veterinary pathologist. After two weeks in formalin, harvested brains were sliced to prepare slides oriented to optimize the cross-sectional orientation of the proximal intracranial arteries. Slides were prepared after hematoxylin and eosin (H&E) staining. Light microscopic analysis was performed to assess the presence of ICAD.(17) As with vwMRI evaluation, basilar and internal carotid artery segments were each rated by the pathologist according to specialty standards as having no, mild, moderate, or advanced ICAD based on presence and severity of histological findings of atherosclerosis: arterial wall thickening, smooth muscle hypertrophy, inflammatory cell infiltration, neointimal formation, lipid deposition, and lumen remodeling.

Statistical analysis was performed on a by-segment basis. To best evaluate samples of these sizes in which normal distribution cannot be assumed, two-tailed Wilcoxon-Mann-Whitney ranked sum tests were performed to compare disease burden among rabbit subtypes. Analysis was performed when considering disease burden in a binary fashion and also on the ordinal severity scale. Sensitivity and specificity were calculated to assess diagnostic accuracy of vwMRI. Statistical analysis was performed using R (R Foundation for Statistical Computing, Vienna, Austria).

Results

17 rabbits and 51 segments underwent evaluation, including 5 WHHL (24.0-34.4 months at euthanasia, mean 28.7 ± 4.3), 4 ApoE knockout (37.8-46.8 months, mean 42.8 ± 4.2), and 8 NZW rabbits (7.1-27.6 months, mean 12.5 ± 7.7). vwMRI and histopathology images

representative of normal vessels and severe disease are provided in Figures 1 and 2, respectively. Table 2 lists vwMRI and pathology results for each animal. 5 (33.3%) vessel segments had ICAD identified on pathology (2 mild, 2 moderate, 1 severe) in WHHL animals, while 6 (50.0%) ApoE knockout vessel segments had lesions (5 mild, 1 moderate). No lesions were identified in NZW control animals. Sensitivity and specificity of vwMRI for detection of ICAD were 68.985.5% and 987.5.2%, respectively, driven by 2 false positives and 5 false negatives. When excluding cases found to have mild disease on pathology to assess vwMRI accuracy for detecting moderate to severe ICAD lesions, sensitivity improved to 100% with unchanged specificity.

Two-tailed Wilcoxon-Mann-Whitney ranked sum tests confirmed that disease model animals had lesions more frequently than NZW animals ($U=216$, $z=2.56$, $p=0.010$). There was no statistical difference in presence of any ICAD between WHHL and ApoE knockout animals ($U=75$, $z=-0.708$, $p=0.478$). The observed presence of moderate or advanced disease noted in WHHL rabbits compared to ApoE knockout rabbits was not statistically significant ($U=41.5$, $z=0.278$, $p=0.780$).

Discussion

ICAD causes more ischemic strokes worldwide than any other etiology.(1–3) In general, it is thought that atherosclerosis develops due to systemic processes that cause lesions to develop near points of stress, such as bifurcations or turns in vessels. However, this scheme has been best validated in the systemic circulation, with little investigation to date examining the pathophysiology of ICAD.(20,21) Intracranial and extracranial arteries are derived from different germ cell layers and have different molecular and anatomic features, so it cannot be assumed that atherosclerosis in these two vascular beds involves identical pathophysiological processes.(22–

24) Little investigation to date has explored the differences between ICAD and extracranial atherosclerosis, but ICAD management strategies still largely mirror those for extracranial disease, ignoring these potential differences.

To develop improved treatment algorithms, better understanding of ICAD pathophysiology and natural history is needed. However, such data cannot be effectively acquired from human investigations alone. Most ICAD is diagnosed at very late stages, and confounding from other diseases of late age, and the treatment of these diseases, often occurs. Additionally, as mentioned above, histopathologic confirmation cannot be performed without unacceptable morbidity. To effectively study ICAD in a longitudinal way that allows characterization of the full course of the disease, we must rely on an effective animal model.

Many insightful studies of atherosclerosis have been performed in rabbits, investigating peripheral and coronary disease.(13) Their size makes both open and endovascular surgical procedures possible, allowing for superior translatability to humans than rodent models. They are large enough for high resolution noninvasive imaging, and their lack of a rete mirabile allows for endovascular access to the cerebral circulation unlike other large animals such as swine.(13) Despite the utility of rabbit models, much less research has been performed on intracranial vessels.

WHHL rabbits have previously been demonstrated to develop ICAD; these animals lack low density lipoprotein (LDL) receptors, which causes them to predictably develop atherosclerosis.(10,11,13,25,26) WHHL research led to the pathophysiologic cause of familial hypercholesterolemia, but this finding suggests they may be a suboptimal model for ICAD.(11) Familial hypercholesterolemia patients typically do not develop intracranial disease despite their profound systemic atherosclerotic burden, and nearly all ICAD patients do not have familial

hypercholesterolemia, so this model could offer spurious vessel biology data with poor correlation to the preponderance of humans with ICAD.(27–29) As a practical consideration, WHHL rabbits have poor health in general, and ICAD has only been described in them after induction of hypertension, which can further complicate their care and limit longevity.(14,15) Coupled with logistical difficulties that make steady supply of WHHL animals impossible, the need for a better ICAD model becomes apparent.(16)

Other avenues exist for inducing atherosclerosis in rabbits. Some investigators have induced disease in NZW rabbits by feeding them atherogenic diets, but such an approach has not proven effective for ICAD.(13,17) ApoE knockout has been used for disease investigation in a variety of species, and a rabbit strain that can be selectively bred was created in recent years.(18,19,30,31) This current investigation sought to evaluate such ApoE knockout rabbits against WHHL rabbits when evaluated with both vwMRI and histopathology. Both WHHL and ApoE knockout animals were found to harbor ICAD lesions. No ICAD was identified in NZW control animals. A nonsignificant trend was noted for more advanced disease to be seen in WHHL rabbits compared to ApoE knockout specimens. This research showed that vwMRI studies were able to reliably detect ICAD lesions, particularly those that proved to be moderate or severe on pathology.

While both WHHL and ApoE knockout animals demonstrated ICAD, as desired for an animal model, limitations of the current analysis bear noting and should be addressed in future investigations. First, none of the WHHL animals underwent induction of hypertension as has been described previously.(15) This may limit comparison to previous studies of ICAD in WHHL rabbits. In comparing WHHL and ApoE knockout animals, ApoE knockout animals were fed an atherogenic diet. Both WHHL and ApoE knockout animals were older than their

NZW counterparts. While NZW animals are not expected to develop ICAD, closer age matching could be performed in future studies. Additionally, earlier stages of ICAD in younger animals could also be investigated to provide a more complete view of the disease course.

While both WHHL and ApoE knockout rabbits were found to harbor ICAD lesions, ApoE knockout rabbits likely represent the better option given supply limitations with WHHL animals and the potential for poor fidelity to human ICAD. In addition to the need for more investigation generally, future efforts should focus on means for accelerating the progression of ICAD in ApoE knockout rabbits to allow for more efficient investigation.

Conclusion

ICAD can be reliably produced and detected using 3T vwMRI-compatible rabbit models. Mature WHHL and ApoE knockout rabbits demonstrated ICAD. Further analysis is needed to better characterize development and progression of disease in these animal models. vwMRI sequences can be used to analyze the disease in these model animals with high sensitivity and specificity, particularly with moderate to severe disease. Given comparable findings between the two models, ApoE knockout rabbits can be considered a suitable alternative to WHHL rabbits given supply constraints and concerns about their translatability to human ICAD.

Figure Captions

Figure 1. Arrowheads demonstrate a normal basilar artery in a wild type NZW rabbit on A) 3D TOF MRA, B) T2 SPACE, C) noncontrast T1 DANTE, D) postcontrast T1 DANTE images. E) Photomicrograph of H&E stained slice at 50X magnification at the corresponding

level demonstrates normal basilar artery wall (arrow) with the brainstem margin at the bottom of the image. Blood products and clot fill the lumen after suboptimal perfusion to clear the vessel.

Figure 2. A) pre- and B) post-contrast T1 DANTE sequences demonstrate enhancing plaque (arrowheads) at the distal basilar artery of a WHHL rabbit, which was rated as severe. C) ~~C)~~ T2 SPACE images of an adjacent slice through the brainstem demonstrates an associated left middle cerebellar peduncle infarct (white arrow). D) Normal and E) abnormal segments of the basilar artery are noted by arrowheads on 3D TOF MRA, with the abnormal segment corresponding to that demonstrated on T1 DANTE images. F~~D~~) Photomicrograph of ~~two slices of~~ the equivalent segment of the distal basilar artery stained with H&E and magnified 50x demonstrates asymmetrical hypertrophic neointima (NI) formation ~~demarcated by black arrows~~. Composed of smooth muscle cells, extracellular matrix with lipid deposition (*) and smooth muscle hypertrophy, findings are consistent with atherosclerotic plaque formation that was rated advanced.

Table 1: vwMRI Scan Parameters

Sequence	3D TOF	T1w SPACE Pre	3D MPRAGE	DWI	T2w SPACE	T1w SPACE Post
Orientation	Axial	Obl-Axial	Obl-Axial	Obl-Axial	Obl-Axial	Obl-Axial
Resolution (mm)	0.3	0.2	0.2	0.7	0.2	0.2
Slice Thick (mm)	0.3	0.2	0.2	2.0	0.2	0.2
Number of Slices	36 per slab 3 slbs	240	160	64	240	240
TR/TE (ms)	18/4.5	550/26	8.9/3.9	5000/74	550/26	550/26
Preparation	Sup Sat	DANTE Prep =150ms	IR Prep TI=350 ms	b=0/1000s/mm^2 Fat Saturation	DANTE Prep =150ms	DANTE Prep =150ms
Scan time (m:s)	4:41	8:34	1:24	2:50	8:15	8:34

Table 2: Animal, Vessel Segment Findings

					ICAD Severity on vwMRI			ICAD Severity on Pathology		
Animal	**Strain**	**Age(mos)**	**Sex**	**Mass(kg)**	**Right ICA**	**Left ICA**	**Basilar**	**Right ICA**	**Left ICA**	**Basilar**
1	WHHL	28.0	M	2.66	None	None	None	None	None	Mild
2	WHHL	31.7	F	3.41	None	None	Moderate	None	None	Moderate

3	WHHL	24.0	M	2.68	Mild	Mild	Advanced	None	None	Advanced
4	WHHL	34.4	F	3.31	None	None	None	None	None	None
5	WHHL	25.6	M	3.8	None	None	Moderate	Mild	None	Moderate
6	ApoE	47.0	M	4.4	None	None	None	None	None	None
7	ApoE	38.3	M	4.79	Moderate	None	None	Moderate	None	None
8	ApoE	36.3	M	4.8	None	None	None	None	Mild	Mild
9	ApoE	46.1	M	4.33	Mild	Mild	None	Mild	Mild	Mild
10	NZW	8.1	F	3.49	None	None	None	None	None	None
11	NZW	10.6	F	3.37	None	None	None	None	None	None
12	NZW	7.1	F	4.28	None	None	None	None	None	None
13	NZW	21.5	F	2.9	None	None	None	None	None	None
14	NZW	9.9	F	3.56	None	None	None	None	None	None
15	NZW	7.7	F	3.59	None	None	None	None	None	None
16	NZW	7.7	F	2.4	None	None	None	None	None	None
17	NZW	27.6	F	4.33	None	None	None	None	None	None

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