Inflammatory Atherosclerotic Plaque Identification by SPECT/CT Imaging of LFA-1 using $[^{111}\text{In}]$ In-DANBIRT in a Novel Dyslipidemic Rat Model

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Research Article

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Abstract

Introduction

Atherosclerosis is prevalent globally, closely associated with dyslipidemia and other metabolic dysfunction. Early diagnosis of atherosclerosis is challenging due to limited diagnostic capabilities that need to be expanded with animal models with enhanced vascular biology like rats. Our previous research showed [111In] In-DANBIRT has potential as a diagnostic tool for detecting atherosclerosis in mice. The primary aim of the present study is to evaluate [111In] In-DANBIRT in a novel atherosclerotic rat with early and late-stage atherosclerosis and metabolic disease.

Methods

We characterized metabolic and body composition differences in these novel dyslipidemic rats under different diets using serum chemistry and DEXA scan, respectively. We performed 1-hour post injection in vivo molecular imaging of ApoE knockout, Lean Zucker (LZ) male rats at baseline and followed them into 10 weeks of either normal or high fat/cholesterol diet implementation (22 weeks of age).

Results

We identified significant differences in body composition and metabolic changes in ApoE knockout rats compared to ApoE wildtype rats. Our findings indicate an increased uptake of [111In] In-DANBIRT in ApoE knockout, lean Zucker (LZ) rats, particularly in the descending aorta, a location where early-stage atherosclerosis is commonly found. Our findings however also revealed that the ApoE knockout, Zucker Diabetic Fatty (ZDF) model has high mortality rate, which may be attributed to alterations of critical enzymes involved in regulating metabolism and liver function.

Conclusion

Our results are highly encouraging as they demonstrated the potential of [111In] In-DANBIRT to detect early-stage atherosclerosis in rats that might otherwise go unnoticed by other methods, showcasing the high sensitivity of [111In] In-DANBIRT. Our future studies will aim to establish a viable T2D atherosclerosis model in rats with more advanced stages of the disease to further demonstrate the reliability of [111In] In-DANBIRT as a diagnostic tool for patients in all stages of atherosclerosis.

Introduction

Atherosclerosis, a prevalent global cardiovascular disease, is typified by the formation of vascular plaques resulting in occlusive events[1, 2]. It is frequently correlated with chronic comorbidities such as Type 2 Diabetes Mellitus (T2D), with patients of T2D displaying an accelerated pathogenesis [3]. Current clinical imaging diagnostics primarily focus on observing alterations in vascular wall structure, which usually become detectable only in advanced stages of the disease, thereby limiting the effectiveness of
early clinical intervention [4]. Thus, there is a critical need for the development of diagnostic tools that can accurately detect early atherosclerosis.

Our previous work has shown that [111In] In-DANBIRT can effectively identify inflammatory activity surrounding the development of atherosclerotic plaques in apolipoprotein E (ApoE) knockout (ApoE KO) mice on a high-fat diet (HFD), suggesting its potential as an ideal diagnostic tool for atherosclerosis [5]. However, there has been ongoing debate regarding the translational relevance of this mouse model of atherosclerosis [6–8]. As such, we argue that there is an urgent need for an animal model that more closely replicates human pathology and provides sufficient spatial resolution for molecular imaging.

Rats have garnered increasing interest as an economical preclinical model that mimics key characteristics of atheroma; however, no model to date fully encapsulates the range of early vascular remodeling and T2D dysfunction observed clinically [9]. For instance, while the Zucker Diabetic Fatty (ZDF) rat is a well-studied model of T2D that reproduces features of human T2D, ZDF rats do not naturally develop atherosclerosis, but rather only demonstrate mild hyperlipidemia under a high-fat diet (HFD) [10, 11]. Therefore, in this short communication paper, we primarily aim to create rat models that replicate early-stage atherosclerosis by utilizing a novel CRISPR/Cas9 approach to knockout ApoE in Lean Zucker rats (LZ). We also applied the same technique to ZDF rats to attempt to generate a rat model that represents a more severe stage of atherosclerosis, which would provide a point of comparison to the LZ model. We utilized [111In] In-DANBIRT SPECT/CT imaging to further demonstrate its potential in accurately characterizing atherosclerosis regardless of severity.

**Materials and Methods**

**Animal Model Development**

A colony of Apolipoprotein E knockout (ApoE KO) rats was established on the Zucker Diabetic Fatty (ZDF) background as previously detailed, utilizing the CRISPR-Cas9 system in combination with superovulation protocols [24, 25] (Fig. 1). Lean Zucker (LZ) rats served as control subjects relative to ZDF rats. ApoE wildtype rats, sourced from Jackson Laboratories, were used to establish control colonies for both ApoE+/+, LZ and ApoE+/+, ZDF rats. The experiment was divided into four distinct groups: ApoE-/-;LZ, ApoE+/+,LZ, ApoE-/-;ZDF, and ApoE+/+,ZDF. Rats were maintained under a 12-hour light/dark cycle and received either a standard diet or a high-fat diet (HFD) (20% protein, 0.21% cholesterol, 21% fat (D12079B, Research Diet)) for 10 weeks, starting at 12 weeks of age. Each group consisted of six rats, randomized post-weaning. Following the final data collection point, euthanasia was carried out in accordance with The American Veterinary Medical Association (AVMA) Euthanasia Guidelines using carbon dioxide inhalation (displacement rate of 10–30% per minute). Any rats that died during the experiment were excluded from further analysis.

**Body Weight Analysis**
Dual energy x-ray absorptiometry (DEXA) scans were conducted to determine lean muscle mass and body fat mass, as well as their corresponding percentages (GE Medical Systems). Following quality control validation using a QC template plate specific for the Lunar PIXImus DEXA scanner, rats were positioned on the measurement plate under continuous isoflurane-induced anesthesia (3% induction, 1–2% maintenance). Data regarding area (cm2), lean muscle and fat mass (grams), and percentages per region of interest were collected. Two scans were performed per rat (upper body and abdominal scan) with five rats per group. Abdominal circumference was measured manually using a measuring tape.

Serum Chemistry Analysis

Whole blood was collected from the tail vein of each rat at baseline and post-diet intervention. The blood was placed into serum separator tubes (BD Biotechnologies) and centrifuged following the manufacturer’s instructions, then stored at -80°C until required. Serum chemistry analysis was conducted using the Vetscan system (Zoetis), which facilitates serum chemistry, electrolyte, and acid-base quantification. Serum was used at a standard dilution according to manufacturer’s guidelines (six rats per group).

Radiolabeling of DOTA-Alkylamino-NorBIRT (DANBIRT) with 111Indium (111In)

Radiolabeling methods were followed as previously described [15]. Briefly, the quality of radiolabeling was verified using Instant Thin-Layer Chromatography (ITLC) with 4mM Diethylene Triamine Pentaacetic Acid (DTPA) on a silica gel strip using 0.9% NaCl. High-performance liquid chromatography (HPLC) was performed with 4mM DTPA at a flow rate of 1mL/min. Radiolabeled DANBIRT demonstrated an incorporation yield > 95% and radiochemical purity > 90%. DANBIRT was radiolabeled with 111In (GE Radiopharmaceutical Department) at a specific activity of [625mCi/pM] and concentration of 1ug:1uL. For each 4mCi of 111In, 6µg of ANBIRT was used. Radiolabeled DANBIRT was adjusted to a pH range of 4.0–4.5 using ammonium acetate.

[111In] In-DANBIRT SPECT Imaging

1-hour post-injection imaging was carried out in ApoE-/-;LZ and ApoE+/+,LZ rats at baseline and following 10 weeks of either a normal or HFD. Methods were performed as previously published [15]. Approximately 900uCi of [111In] In-DANBIRT was administered via tail vein injection. The NanoSPECT/CT® Bioscan was utilized while animals were under deep anesthesia with 3% Isoflurane. Regions of Interest (ROI) were determined for the muscle, carotid arteries, aortic arch, and descending aorta using VivoQuant 2.00 (invICRO, Boston, MA). ROI were extrapolated from our predetermined phantom image scan and adjusted. Radioactive activity per volume was normalized to muscle (background tissue). Activity was decay corrected and compared within and among groups (six rats per group).

Statistical Analysis
All statistical analyses were performed using two-tailed Student's t-tests, one-way and two-way factor ANOVA with Tukey's post-hoc test, as applicable. The mean difference in quantitative uptake of Percent Injected Activity per gram of tissue (%IA/gr) normalized to muscle of radiolabeled DANBIRT was compared. GraphPad Prism software (Montreal, Quebec, Canada) was used for all statistical analyses. Data are represented as mean ± standard error (SE) unless otherwise specified. Sample sizes ranged from 3–6 rats per group, as detailed in each assay. Statistical significance was established at *: p < 0.05, **: p = 0.001–0.01, ***: p = 0.0001–0.001, ****: p < 0.0001.

Results

Metabolic and obesity evaluation of the ApoE KO rat

Our studies revealed significant differences between male ApoE\(^{-/-}\),LZ and ApoE\(^{-/-}\),ZDF rats (Fig. 2). By measuring abdominal circumference, body weight, and employing dual energy x-ray absorptiometry (DEXA), we were able to characterize these differences in body composition (Fig. 2A-D). We found that ApoE\(^{-/-}\),LZ rats were nearly as heavy as ApoE\(^{-/-}\),ZDF rats (Fig. 2C). However, ApoE\(^{-/-}\),ZDF rats appeared to have significantly decreased percentage of muscle mass, possibly owning to their severely developed T2D phenotype (Fig. 2A). ApoE\(^{-/-}\),LZ rats on a HFD gain a substantial amount of weight (Fig. 2G). Yet, even though these ApoE\(^{-/-}\),LZ rats are as heavy as ApoE\(^{-/-}\),ZDF rats, they do not have nearly as much abdominal fat as ApoE\(^{-/-}\),ZDF rats (Figure E, F).

After identifying the differences in body composition, we wanted to evaluate changes on a molecular level, especially how metabolic and biological enzymes were altered in rats of different genotype. Therefore, as part of our initial evaluation we performed serum chemistry analysis. Our findings indicate that liver changes were among the most significantly observed (Fig. 3A-C). We found that ApoE\(^{-/-}\),ZDF rats had significantly higher levels of liver enzymes including Alkaline phosphatase (ALP) and Amylase compared to ApoE\(^{-/-}\),LZ rats (Fig. 3A). We found that one of the most used markers for liver damage, Alanine transaminase (ALT), is also increased in ApoE\(^{-/-}\),ZDF rats. In addition to liver enzyme changes, serum chemistry also revealed significantly higher glucose and blood urea nitrogen (BUN) in ApoE\(^{-/-}\),ZDF rats. Altogether, ApoE\(^{-/-}\) rats had a vastly different serum chemistry profile compared to ApoE\(^{+/+}\) rats (Fig. 3B-C), with liver enzymes and glucose were among the most notorious differences between these genetic variants. Same differences were observed between LZ and ZDF rats regardless of their ApoE presence.

ApoE KO, ZDF rats die under HFD

The rationale for using ZDF rats is that they bear the fa mutation in the Leptin receptor. Rats with the phenotype Lepr fa/fa are hyperphagic and present with hyperglycemia and insulin resistance like human T2D. The ApoE\(^{-/-}\),ZDF rats were developed as previously described as the first successful CRIPR/Cas9-mediated gene editing of the Zucker rat achieving complete knockout of the ApoE protein. This genetic
Modification resulted in early lipid deposition in the aortae of these animals when fed with HFD. We proposed to use the novel minimally invasive imaging modality for SPECT imaging using $^{[111}\text{In}]$ In-DANBIRT as a novel imaging pre-clinical tool to characterize the vascular phenotype in this novel rat model with the potential to further our understanding of how diabetes impacts the progression of vascular disease. Figure 4 shows that almost all ApoE$^{-/-}$,ZDF rats died. Our Kaplan Meier survival curve shows ApoE$^{-/-}$,ZDF rats die as early as 3 weeks after HFD implementation (Fig. 4). None of the ApoE$^{-/-}$,ZDF rats lived after 5 weeks of HFD. Contrastingly, around 40% of ApoE$^{-/-}$,ZDF rats on a normal diet also die. None of the ApoE$^{+/+}$,ZDF controls died, meaning mortality is specific to our ApoE deficient rats.

ApoE KO, LZ rats under a HFD show high $^{[111}\text{In}]$ In-DANBIRT uptake in early-atherosclerotic prone vessels. $^{[111}\text{In}]$ In-DANBIRT images were taken on ApoE$^{-/-}$,LZ rats after 10 weeks of HFD induction. Representative images of the ghost image markup were shown in Fig. 5A and 5B, with and without ROI. Image showing the descending aorta was taken from a different angle shown in Fig. 5C. Representative images show ROIs normalized for background muscle uptake, compared to baseline (without diet induction) (E) and 10 weeks of normal diet (F) (Fig. 5E-F). We quantified the intensity of the $^{[111}\text{In}]$ In-DANBIRT and found that LZ rats exhibited significantly higher $^{[111}\text{In}]$ In-DANBIRT uptake in the descending aorta (Fig. 5D). Aortic arch and common carotids also show an increase in $^{[111}\text{In}]$ In-DANBIRT uptake, but the results are not statistically significant (Fig. 5D). We also performed radiochemical purity and radiolabeling yield for quality control before injection (Supplemental Fig. 1).

**Discussion**

Increase in ALP and ALT has been most closely associated with liver damage in metabolic disease evolution[12, 13]. Increase in liver enzymes has shown to be directly correlated to the development of diabetes phenotype in rats [14, 15]. Similar observations have been made in diabetic patients[16]. Thus, it appears to us that the significant increase in serum ALP and ALT levels in ApoE$^{-/-}$,ZDF rats, combined with higher glucose levels, suggests that ZDF rats may have developed more severe T2D phenotypes early in the animal model development. This indicates severity particularly in response to HFD induction, characterized by increased liver damage and worse hyperglycemia. In addition, elevated levels of BUN in ApoE$^{-/-}$,ZDF rats indicates possible kidney damage compared to ApoE$^{-/-}$,LZ rats [17, 18]. Serum chemistry and glycemia are the parameters we evaluated to correlate the already known lipid level increase for dyslipidemia in ApoE KO rats. These parameters point towards the increased risk of atherosclerotic development as it occurs in the clinic. Our aim is to evaluate the atherosclerotic lesions externally without having to sacrifice the animal. SPECT/CT imaging allowed us to follow the development of vascular atherosclerotic lesions externally in vivo and non-invasively in a time-dependent manner.

HFD induces the development of metabolic syndrome, which consists of the production of reactive oxygen species (ROS), atherogenic-dyslipidemia and contributes to the development of non-alcoholic
fatty liver disease[19]. We believe a possible explanation for the increase in mortality in our rat model due to a link between ALP and the promotion of high production of ROS[20]. In hypertensive patients, fibrosis and perivascular inflammation correlates with increased ROS production and arterial calcification. This promotes vascular damage in both the micro- and macro-circulation in different organs such heart & kidney[21]. ZFD rat models have shown higher oxidative stress in myocytes caused by mitochondrial disruption[22]. The overaccumulation of ROS tends to conduct the mitochondria conduct autophagy and then leads to apoptosis releasing this species to the media and promoting inflammation[23, 24]. ZDF rats under a HFD are known to develop hypertension with lower mitochondrial biogenesis followed by autophagy[25, 26]. Diabetic cardiomyopathies are associated with fat intake and high concentration of oxidative stress, two of the main characteristics of our model[27]. Serum concentrations of ALT predict the development of both liver and endothelial dysfunction[28]. ALT is more specific to the liver, high-altitude exposure promotes its accumulation[29].

All ApoE⁻/⁻,LZ rats regardless of the diet implemented lived through our studies (Fig. 4). We compared the data from these males to some of our original data we have in parallel published studies from females and there was a significant difference in mortality in such groups. Due to our findings, ApoE⁻/⁻,LZ rats were our focus and showed the most promising results due to this incidental finding. Potentially, ZDF male rats could die sooner than female ZDF rats because there is a higher concentration of glutathione (GSH) by overproduction in females rather than in male ZDF models[22, 27]. Together with the disease model mortality, high altitude and dehydration risk with our shipping to Albuquerque, NM increased the mortality of our male rats specifically ApoE⁻/⁻,ZDF. High altitude and less GSH production would contribute to more urea concentration, being detrimental for our rats health[27]. We aim to explore these sex-specific effects in mortality in this rat model in future studies.

Multiple studies have identified similar findings and characterized descending aorta atherosclerosis as the indicator or initial stage of a more severe stage of atherosclerosis in both animal models and humans[30–33]. This is particularly exciting for us because it demonstrates the possibility and potential of using [¹¹¹In] In-DANBIRT as a powerful and precise clinical diagnostic tool that enables clinicians to identify early atherosclerosis patients with small plaque deposits that would otherwise go undetected by other currently available tools[30–32]. Khoury et al. pointed out that descending aorta plaques have a significantly higher sensitivity in predicting coronary artery disease (93%) compared to ascending aorta (37%)[33]. Our findings suggested that [¹¹¹In] In-DANBIRT may have the potential to diagnosis and therefore prevent the progression of early atherosclerosis, potentially saving millions of patients in the future from severe cardiovascular events.

Conclusion

[¹¹¹In] In-DANBIRT is a reliable imaging tool with translational potential for external longitudinal molecular imaging in preclinical studies. We have shown that this imaging modality can detect even the slightest increase in immune cell activity in ApoE⁻/⁻,LZ rats, demonstrating its unique potential to detect
early-stage atherosclerosis. Despite our efforts to use the ZDF rat, which is a novel translational model for us to understand clinical atherosclerosis, it has been demonstrated that they cannot be studied in detail in our current stage due to their high mortality rate after HFD induction. Thus, to further demonstrate $^{111}\text{In}$-DANBIRT's superior capability and clinical potential under more advanced atherosclerosis conditions, our future research will concentrate on developing a novel rat model that better mimic chronic atherosclerosis patients. Our current research is significant because it identifies $^{111}\text{In}$-DANBIRT as one of the few diagnostic tools capable of detecting atherosclerosis in its earliest stages, bring us one step closer to diagnosing atherosclerosis plaque in patients prior to life-threatening complications.

**Declarations**

**Ethics approval and consent to participate**

All animal experiments were approved by the ethics committee of the University of New Mexico Institutional Animal Care and Use Committee (IACUC) and Animal Welfare Committee. Approved protocol number: 18-200791-HSC. All live vertebrate studies were carried out ethically in accordance with relevant guidelines and regulations at the University of New Mexico.

**Data availability**

The data is available in a MENDELEY DATA repository: Mota Alvidrez, Roberto (2023), “Inflammatory Atherosclerotic Plaque Identification by SPECT/CT Imaging of LFA-1 using $^{111}\text{In}$-DANBIRT in a Novel Dyslipidemic Rat Model”, Mendeley Data, V1, doi: 10.17632/6rzygw6c5c.1

**Conflict of interest**

The authors declare that no significant conflicts of interest exist.

**Author contributions**

ZL, MJC, RIMA conceived of the study, design, coordination and writing of the manuscript. RIMA performed the experimental studies. TAD participated and aided in the in vivo studies. ZL, MJC provided support and resources for the experimental studies. ZL, RIMA performed the statistical analysis of the data. ZL, MJC provided critical study design and direction, as well as manuscript editing. All authors read and approved the final manuscript.

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References


Figure 1

Graphical abstract showing rat model development aim of the studies. Created with BioRender.
Figure 2

Longitudinal weight and DEXA scan body composition reflect increased abdominal circumference in obese rats compared to lean control rats.

(A-D) Rat body composition was measured by percentage of lean muscle mass (A), fat percentage (B), body weight (C) and abdominal circumference (D). Data demonstrated that ApoE\(^{-/-}\),LZ and ApoE\(^{-/-}\),ZDF rats have similar body weight but mostly differences are observed with ApoE\(^{-/-}\),LZ having higher lean muscle%, less fat% and less abdominal circumference compared to ApoE\(^{-/-}\),ZDF rats at baseline before diet implementation. (E) Representative images of DEXA scans are shown for ApoE\(^{-/-}\),LZ (E) and ApoE\(^{-/-}\),ZDF rats (F). (G) Longitudinal weight measurements are shown comparing ApoE\(^{-/-}\),LZ and ApoE\(^{-/-}\),ZDF rats to their ApoE\(^{+/+}\) counterpart (reference control) under NC and HFD as time progresses during our studies. Two tailed students t-test was performed, and one-way ANOVA were performed respectively. ANOVA results, F: 20.5, p: <0.0001. Data is represented as mean ± SEM. Statistical significance p was set at *:<0.05, **: 0.001-.01, ***: 0.0001-.001,****: <0.0001.
Serum chemistry analysis of dyslipidemic rats. We had previously shown both LZ and ZDF ApoE KO (ApoE^{-/-}) rats develop dyslipidemia under a HFD. Results show our rats also display distinct serum chemistry panels (A-C). Pairwise comparison from both groups show distinctive differences primarily in ALP, amylase, BUN and glucose mostly higher in ApoE^{-/-};ZDF rats (A). We also show that compared to ApoE WT rats under LZ and ZDF controls there is a difference in almost all parameters. Particularly, ApoE^{-/-};LZ rats have higher ALT and glucose compared to age matched ApoE^{+/-};LZ control rats (B). ApoE^{-/-};ZDF rats show almost 5-fold higher ALT, higher sodium, globulin and glucose compared to age matched ApoE^{+/-};ZDF control rats (C). Data is represented as mean ± SD as well as SEM were applicable. Statistical significance p was set at *:<0.05, **: 0.001-.01, ***: 0.0001-.001,****: <0.0001.
Kaplan Meier survival analysis show ApoE\(^{-/-}\),ZDF rats have a short lifespan, particularly under HFD. We performed survival analysis of all rats for our experimental groups. ApoE\(^{-/-}\),ZDF rats under a HFD do not live past 19 weeks of diet implementation (100% mortality). ApoE\(^{-/-}\),ZDF rats under a normal diet (60% lived to the last day of experimental time point) (p value: <0.0001, Chi-square: 98.75). All the other groups outlived up to the last day of diet implementation. Statistical significance p was set at *:<0.05, **: 0.001-.01, ***: 0.0001-.001,****: <0.0001.
**Figure 5**

In vivo $[^{111}\text{In}]$ In-DANBIRT longitudinal SPECT/CT imaging of atherosclerotic disease. ApoE$^{-/-}$,LZ rats fed a HFD exhibit increased from radiolabeled inflammatory cells mostly in the descending aorta. Representative phantom SPECT/CT scan without ROI markup is shown 1-hour post injection of $[^{111}\text{In}]$ In-DANBIRT (A). ROI markups show tissues of interest: muscle, carotid arteries, aortic arch and descending aorta (heart is shown for anatomical references). Uptake in $\mu$Ci/mm$^3$ was normalized to muscle uptake.
High $^{[11]}\text{In}$-DANBIRT uptake from ApoE$^{-/-}$,LZ rats after 10 weeks of HFD (D) compared from baseline (E), 10 weeks of normal diet (F) and HFD (G). ANOVA results, F: 10.13, p: <0.0001. Data is represented as mean ± SEM. Two-way ANOVA with multiple comparisons (Tukey's post-hoc) was performed. Statistical significance p was set at *:<0.05, **: 0.001-.01, ***: 0.0001-.001,****: <0.0001.

Supplementary Files

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- SFigure1.pdf