Chronic kidney disease-associated cardiovascular disease: scope and limitations of animal models

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Chronic kidney disease (CKD) is a heterogeneous range of disorders affecting up to 11% of the world's population. The majority of patients with CKD die of cardiovascular disease (CVD) before progressing to end-stage renal disease. CKD patients have an increased risk of atherosclerotic disease as well as a unique cardiovascular phenotype. There remains no clear aetiology for these issues and a better understanding of the pathophysiology of CKD-associated CVD is urgently needed. Although nonanimal studies can provide insights into the nature of disease, the whole-organism nature of CKD-associated CVD means that high-quality animal models, at least for the immediate future, are likely to remain a key tool in improving our understanding in this area. We will discuss the methods used to induce renal impairment in rodents and the methods available to assess cardiovascular phenotype and in each case describe the applicability to humans. *Cardiovasc Endocrinol* **6:120–127**

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Introduction

Chronic kidney disease (CKD) is the term given to a heterogeneous range of disorders affecting the structure and function of the kidney. It is a global health problem, affecting up to anywhere between 9 and 11% of the world's population [1]. It is driven by a broad range of aetiologies and can progress to end-stage renal disease, a condition that can only be treated by dialysis or renal transplantation. However, populations with CKD pose a major healthcare burden aside from provision of renal replacement therapy, mainly attributable to cardiovascular (CV) morbidity and mortality. Indeed, the majority of patients with CKD will die from cardiovascular disease (CVD) before progressing to end-stage renal disease [2].

Compared with the general population, patients with CKD have a higher burden of traditional CVD risk factors. These in turn behave in a more accelerated fashion, for example, CKD patients have more severe coronary artery atherosclerotic plaque formation compared with the general population [3,4] and the risk of myocardial infarction among CKD patients is twice that of patients without CKD [5]. Similarly, peripheral artery disease [6] and stroke [7] all show increased risk as the estimated glomerular filtration rate begins to fall below 60 ml/min/1.73m^2.

Alongside increased risks of atherosclerotic disease, CKD also leads to a distinctive CV phenotype characterized by prominent endothelial dysfunction, arterial stiffening and calcification along with left ventricular hypertrophy, fibrosis and cardiac dysrhythmia. CKD-associated CVD typically leads to clinical outcomes such as admission for heart failure and sudden cardiac death. It is these clinical events that constitute the leading causes of morbidity and mortality in patients receiving dialysis [8]. Although a number of underlying risk factors and mechanisms for this clinical syndrome have been described including proteinuria [9], anaemia [10], salt retention [11], retention of uraemic toxins [12], inflammation [13] and oxidative stress [14], there remains no clear aetiological framework for these problems. A better understanding of the pathophysiology of CKD-associated CVD is therefore urgently required both to better understand the nature of the risk and to develop novel therapies aimed at reducing the burden of CVD in this population. Although epidemiological, clinical translational and in-vitro studies can provide insights into the nature of disease, the whole-organism nature of CVD-associated CVD means that high-quality animal models, at least for the immediate future, are likely to remain a key tool towards improving our understanding in this area.

Most scientists aspire to a future where the use of cell-based systems and cross-platform bioinformatics approaches makes animal experimentation redundant, but currently rodent disease models provide a unique insight into whole-organism physiology and pathophysiology. However, although animal models of disease remain necessary, all studies should be designed to minimize suffering and in accordance with the 'three R's', that is, 'Replacement' of animals with other methods if possible, 'Refinement' of animal models to maximize data gained

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Table 1 Chronic kidney disease phenotyping in animal models

Model	Method of injury	Species	Strain	Sex differences	Time course	Renal histological findings in animal	Translation to human disease	Other considerations	References
5/6 nephrectomy	Renal mass reduction	Rat/mouse	Multiple	Males more susceptible	0–12 weeks	Glomerulosclerosis, mesangial expansion, tubulointerstitial fibrosis	Model for hyperfiltration injury, e.g., renal cancer nephrectomy patients	Mice (especially C57BK/6) highly resistant Surgically complex Removed kidney is good control	Chow and colleagues [21–23]
Adenine	Tubular injury	Rat/mouse	Multiple	Males more susceptible	6 weeks	Tubulointerstitial fibrosis, vascular calcification	Mimics CKD-MBD	Simple Rapidly progressive	Yokozawa and colleagues [24–28]
Aristolochia	Tubular injury	Rat/mouse	Multiple	None known	1–6 weeks postdose	Tubulointerstitial fibrosis, potentially some podocyte damage	Aristolochia nephropathy	Simple Toxic nephropathy rare cause of CKD in humans	Debelle and colleagues [29–31]
Folic acid	Tubular injury	Rat/mouse	Multiple	None known	1–6 weeks postdose	Tubulointerstitial fibrosis, potentially some podocyte damage	Cyclosporine induced interstitial nephritis	Toxic nephropathy rare cause of CKD in humans	Doi and colleagues [32–34]
DOCA-salt	Renal mass reduction, vasoconstriction, hypertension	Rat/mouse	Multiple rat, C57 mice	More severe in males	4 weeks	Glomerulosclerosis	Hypertensive CKD	Proteinuria, hypertension, low renin	Mohammed-Ali and colleagues [35–38]
Anti-Thy1	Immune-mediated injury	Rat/mouse	Multiple	None known	Immediate to 3 weeks	Mesangialnecrolysis, mesangial expansion, occasional crescents	General model of glomerulonephritis with a predominant mesangial component	Needs nephrectomy to produce longer term phenotype	Ishizaki and colleagues [39–41]
Unilateral ureteric obstruction	Ligation of single ureter	Rat/mouse	Multiple	None known	0–2 weeks	Hydronephrosis, tubulointerstitial fibrosis	Ureteric obstruction not common cause of CKD	No change in functional readouts Allows for internal control	Klahr and colleagues [42–44]

CKD, chronic kidney disease; DOCA, deoxycorticosterone acetate; Thy1, thymocyte.

Table 2 Cardiovascular phenotyping in animal models

Clinical CV phenotype	Measure in animals	Advantages	Disadvantages	References
Blood pressure	NIBP	Simple	Not reproducible Complicated by anaesthetic	Malkoff and colleagues [45–48]
	Terminal IBP	Simple	Sacrifices animal	
	Telemetry	Reliable and continuous	Surgery required	
Arterial stiffening and calcification	PWV	Can be done noninvasively	Cannot differentiate atherosclerotic disease from medial fibrosis and calcification	Shobeiri and colleagues [49–52]
	Endothelial function	Allows for mechanistic approach to CKD-CVD	Whilst plays a part in human disease – not routinely measured in clinic practice	Hasdan and colleagues [53–55]
	Histology/transcriptomics	Allows for mechanism discovery		Iyer *et al.* [54]
Atherosclerotic disease	Endothelial function	As above	As above	As above
	Histology	Better understanding of underlying pathophysiology		Shobeiri *et al.* [49] Terai *et al.* [56]
Left ventricular hypertrophy	Heart weight	Simple	Coarse measurement	Iyer *et al.* [54]
	In-vivo imaging	Functional read outs, more physiological. Read-outs can easily compare with human clinical practice	Technically challenging	Zhang and Kompa [22] Hu *et al.* [57]
Heart failure	In-vivo imaging	As above	As above	As above
	Ex-vivo functional studies	Allows for easy variation of environment with functional readouts	Can only partially recreate true internal physiology	Sviglerová *et al.* [58]
	Histology	Allows for investigation of final common pathway	Sacrifice of animal	As above

CV, cardiovascular; CKD, chronic kidney disease; CVD, cardiovascular disease; IBP, invasive blood pressure monitoring; NIBP, Non-invasive blood pressure; PWV, pulse wave velocity.

per animal and 'Reduction' of animals used where possible [15].

There is a long history of modelling chronic renal injury in rodents [16]. These models can be used to (i) simulate specific diseases that cause CKD, for example diabetes [17] or systemic lupus erythematous [18]; (ii) examine the final common fibrosis pathways of progressive CKD; or (iii) to investigate CKD complications such as CKD-associated CVD. It is this third aim that will be the focus of this review.

Although atherosclerotic disease and the consequences of athero-occlusive events have been modelled in animals in the context of renal injury (e.g. ApoE KO [19] or coronary artery occlusion in rat models [20]), we focus on models aimed at investigating the distinctive CV phenotype of CKD-associated CVD in this review. We will discuss the methods used to induce renal impairment in rodents (Table 1) and the methods available to assess CV phenotype (Table 2) and in each case describe the applicability to humans.

Rodent models of chronic kidney disease

Unilateral ureteric obstruction

The unilateral ureteric obstruction model has been used in rats and mice. One ureter is tied off, causing increased urinary tract pressure proximal to the lesion, followed by an interstitial inflammatory response with subsequent cellular invasion and eventual tubulointerstitial fibrosis and atrophy. The degree of fibrosis is proportional to the length of time that the ureter is tied off [42]. In general, the contralateral kidney in-situ provides a control 'normal' kidney to be analysed histologically alongside the obstructed one. The advantage of this model is that it is technically less challenging than nephrectomy, it is easily reproducible and works in most strains of both mice and rats. The renal damage occurs rapidly, reaching a peak within 7 days. However, although human CKD can be caused by urinary obstruction [43], overall, it is not a major cause of CKD in the adult population, and even when it is, partial rather than complete obstruction is typical [59]. Furthermore, in rodent models, the remaining functioning kidney also goes onto compensate for loss of function of the obstructed kidney. Thus, biomarkers of renal failure, such as serum urea and creatinine or proteinuria, are often not clearly elevated using this model. Efforts to better reflect human disease, for example partial or reversible obstruction, have been explored [44], but are not in widespread use because of their surgical difficulty, therefore limiting the utility of this type of model.

Surgical nephron reduction

Subtotal nephrectomy mimics the consequences of reducing functional renal mass. It is most often used in rats and encompasses two different methodologies. The first is a ligation model, where one kidney is removed,

with consequent ligation of polar branches of the renal artery of the contralateral kidney. In the other method, the rat undergoes a nephrectomy and then roughly 50% of the contralateral kidney is excised 1–2 weeks later. The latter model does not tend to manifest with hypertension, whereas the former does [21]. In both models, the rats develop progressive renal failure by week 2, with renal histology showing progressive glomerular and interstitial fibrosis with renal dysfunction and proteinuria, which is similar to that found in human disease [22]. Conversely, mice are typically resistant to induction of chronic renal injury by nephron reduction [23], although there is considerable interstrain variability [60]. Furthermore, the challenges of carrying out surgery in small animals should not be underestimated [61].

Uninephrectomy deoxycorticosterone acetate/salt models

The mineralocorticoid deoxycorticosterone acetate (DOCA), when administered with a high-salt diet and unilateral nephrectomy, induces renal injury, low renin levels and hypertension in both rat and mouse models. It consists of a 2–6-week period of high salt and mineralocorticoid exposure. The DOCA itself is administered by either subcutaneous pellet insertion or oral supplementation. Animals then develop hypertension, followed by proteinuria, glomerular sclerosis, tubulointerstitial inflammation and fibrosis in keeping with progressive CKD [35], although the severity of the phenotype varies between different rodent strains. For example, 129/SV mice have markedly higher DOCA-induced blood pressure (BP), glomerulosclerosis, interstitial fibrosis and albuminuria compared with C57BL/6 mice [36].

This model has been used to investigate the relationship between the renin–angiotensin–aldosterone system activation and CKD [37] as well as the therapeutic effect of angiotensin-converting enzyme inhibitors [38]. The CV phenotype probably reflects only one of the potential mechanisms (i.e. sodium retention ± oxidative stress) through which kidney injury impacts on the CV system. Therefore, although this approach has been used extensively to study hypertension and the final common pathway of CKD, it may not provide a comprehensive model for investigating the fundamental mechanisms of CKD-associated CVD.

Nephrotoxic models of chronic kidney disease

Nephrotoxic models of CKD are attractive as they typically cause less suffering and are technically less challenging than surgical models. First proposed as a viable CKD model in 1982 [24], adenine, when administered in high quantities, saturates the adenine phosphoribosyl transferase pathway, causing it to be oxidized into 2,8-dihydroxyadenine by xanthine oxidase. 2,8-dihydroxyadenine is renally excreted and precipitates within the tubule, leading to tubulointerstitial inflammation and fibrosis [25]. The adenine model leads to severe CKD, with marked biochemical renal failure and associated vascular calcification [49]. By adjusting the concentration of adenine in rat chow, Shobeiri *et al.* [49] could produce stable CKD at 5, 8 and 11 weeks. Sex plays a key role in this model, with female rats requiring higher adenine concentrations in their diet to achieve the same severity of kidney injury [26]. The model has also been validated in mice [27]. Hyperphosphataemia, secondary hyperparathyroidism, renal osteodystrophy and vascular calcification are prominent in this model [28], a phenotype consistent with the consequences of the CKD-mineral bone disorder (CKD-MB) observed in patients.

A single dose of 250 mg/kg of folic acid causes an acute kidney injury in mice. Animals show the development of crystals within the tubular lumen, causing acute tubular damage, followed by tubulointerstitial fibrosis over 2 weeks [32]. By alkalinizing the urine, using sodium bicarbonate, folic acid crystals can be reduced, but tubular damage still occurs, suggesting that a direct nephrotoxic effect also contributes in this model [33]. Acute damage occurs within 2 weeks, with the chronic fibrosis becoming apparent between 4 and 6 weeks. Biochemical markers of renal impairment occur in parallel with renal fibrosis [34], making this a particularly useful model for studying the consequences of CKD of different severities. This model is increasingly being used; for example Rattanasinganchan [62] used this approach to investigate urinary biomarkers of tubulointerstitial fibrosis.

Aristolochia, when administered to animals (rats at a dose of 10 mg/kg daily subcutaneous injections for 35 days, for mice 3 mg/kg, intraperitoneal, every 3 days for 6 weeks), induces proteinuria, elevated serum creatinine with associated tubular necrosis, atrophy and interstitial fibrosis by day 35 [29,30]. These findings closely resemble those found in Chinese-herb nephropathy in humans, a disease in which aristolochia has been shown to be the causative agent [63]. As such, this is a model of human tubulointerstitial nephritis and has predominantly been used to study the molecular basis of kidney fibrosis rather than to investigate CKD-associated complications. However, given that albuminuria is a feature of this model, some studies have suggested that aristolochia may also lead to glomerular damage, with podocyte effacement noted on electron microscopy [31]. This model has predominantly been investigated in mice (NMRI, FVB, C76BL/6 and C3H/He strains have all been studied), and there is some evidence that there may be a degree of renal recovery after 9–15 weeks [30].

Immunological models

Of the immunological models, the anti-Thy1 model in rats has been most studied. Thy1 is an antigen found on glomerular mesangial cells. When rats are injected with a single dose of either antithymocyte serum (containing

anti-Thy1 antibodies) or a mouse anti-Thy1 monoclonal antibody, animals develop a glomerulonephritis [39]. Histologically, there is initially mesangiolysis with an inflammatory cell infiltrate, followed by mesangial matrix expansion with the occasional extracellular crescent [40]. This process takes roughly 1 week, with subsequent repair from 3 weeks onwards. The renal phenotype of proteinuria, haematuria and renal impairment reflects the abnormalities observed in human glomerulonephritis. Although in humans there is hypertension (as opposed to normotension in animals) and immune complex deposits histologically, these are not observed in the animal model. This model has also been combined with uninephrectomy to produce a glomerulonephritis model more typical of a glomerulonephritis-associated CKD [41]. Note that this model is not viable in mice.

Other models

We have only outlined the most commonly used approaches to induce renal injury in rodents. Many others are described, particularly those aimed at modelling specific diseases; these include spontaneous models of glomerular and interstitial injury such as NZB/W lupus nephritis model [64] or the Sprague-Dawley rat ageing model [65], spontaneously hypertensive rats; [66] genetically engineered models such as the mouse Alports model lacking collagen α 3 (IV); [67] acquired immunological models such as models of antiglomerular basement disease; [68] and acquired nonimmunological models such as radiation nephropathy [69] or cyclosporine nephropathy [70].These approaches have been reviewed extensively elsewhere [16].

Cardiovascular phenotyping

BP is the simplest CV phenotype and is typically elevated in CKD. Noninvasive methods involve the use of a tail-cuff. This can be performed on awake, restrained rodents. However, restraint induces agitation in mice and rats unless they have been suitably conditioned and tail-cuff readings generally correlate poorly with invasive measures [45]. Terminal invasive methods require the use of anaesthetic agents that may interfere with BP readings [46]. Conversely, telemetry devices enable BP measurement during normal activity [47] and represent the gold-standard approach; however, this requires additional animal procedures, the surgery is technically challenging and the equipment is expensive. BP measurements have typically been performed in all the models described above. Hypertension is typically observed in the nephron reduction, DOCA-salt, folic acid and the adenine models; however, there are marked differences between species and strains [48].

Pulse wave velocity (PWV) using ultrasound noninvasively or invasively directly relates to the burden of atherosclerotic disease in both animals [50] and humans [51]. However, it is also a key measure of arterial stiffness in the absence of atherosclerosis and therefore an important characteristic of CKD-associated CVD. PWV is quantified in animals by measuring two pressures a known distant apart with high-fidelity transducers and then determining the delay between waveforms. The adenine model shows increased PWV; this occurs early within 5 days of model induction [52] and continues as the animals vasculature becomes more calcified [71].

Abnormal endothelial function has long been linked to the pathophysiology of CKD-associated CVD with a number of implicated mechanisms including alterations in nitric oxide (NO), asymmetric dimethylarginine and advanced glycation end products [72]. Hypertension is known to cause endothelial dysfunction and in nephrectomy rat models [53] and DOCA-salt models, aberrant levels of endothelial mediators are found [54]. However, the folic acid [73], adenine [74,75] and unilateral ureteric obstruction [76] models have all been used to investigate the mechanisms and potential therapeutics of endothelial dysfunction in CKD. Most simply, these methods are ex-vivo transcriptomic assays; however, there have been some attempts to model in-vivo endothelial function using high-resolution ultrasound to measure flow-mediated vasodilation in both rats and mice in response to different endothelial activators or inhibitors [55,77].

Arterial histology in CKD-associated CVD typically shows medial thickening or fibrosis along with calcification in the smooth muscle layer and this phenotype has been demonstrated in animal models. Initially, the adenine model involved feeding the animals a high-phosphate diet that markedly reduced the animals' weight and confounded results; however, an updated model involving a high initial adenine dose combined with a normal diet has resolved this issue [56]. However, in nonadenine models, combining a high-phosphate diet with the existing model can heighten the calcific phenotype [49]. Histopathology can also indicate accelerated plaque formation when examining models of atherosclerotic disease in the context of chronic renal injury.

Functional cardiac imaging is also possible in small animals. Patients with CKD-associated CVD typically have left ventricular hypertrophy, ventricular dysfunction and cardiac fibrosis. Teams such as Zhang *et al.* [22] have utilized echocardiography to analyse systolic and diastolic functioning of the hearts in animal models of renal injury, observing impairments in both. Cardiac MRI has also been used to illustrate cardiac hypertrophy and fibrosis in rodent models of CKD [57].

Others have shown ex-vivo functional changes for example, decreased contractility in a subtotal nephrectomy model in rats [58]. Cardiac histology (and weight) can also be used to demonstrate increased heart size and fibrosis. For example, the DOCA-salt model (rats and mice) also shows a cardiac phenotype in keeping with

hypertension with increased heart size and cardiac fibrosis [54].

To what degree the above animal models of chronic kidney injury recapitulate the CV phenotype, other than hypertension and left ventricular hypertrophy, observed in patients has been explored inadequately in all except a few cases. However, some of the above models do show vascular features suggestive of some aspects of arterial disease observed in CKD and are worth additional comment.

For example, the adenine model, alongside hypertension, mimics the CKD-MBD with evidence of arterial calcification and hyperphosphataemia and secondary hyperparathyroidism. Evidence from this model suggests that vascular smooth muscle cells transform into an osteochondrogenic-like phenotype [78–80].

Reduced NO bioavailability has long been suggested to underlie vascular pathology in CKD. Rats that have undergone subtotal nephrectomy develop a worsening systolic function and cardiac fibrosis (both features of CVD-associated CKD) when treated with a low dose of a NO synthase inhibitor, providing support to this theory [81]. Similarly, a nephrectomy rat model has been used to highlight the role of the uraemic toxin indoxylsulphate in cardiac fibrosis, as well as test out monoclonal antibodies against cardiotonic steroids implicated in uraemic cardiomyopathy [82,83].

Therefore, the evidence for the mechanisms underlying the kidney–vascular link remains sparse and certainly there is no comprehensive understanding of the pathological pathways leading to the development of CKD-associated CVD. This will not only require induction of a range of different models of CKD but the systematic characterization of the CV phenotype using whole-organism physiological measures, imaging and interrogation of isolated tissues across multiple time points.

General considerations when designing experiments on the basis of animal models of chronic kidney disease-associated cardiovascular disease

A number of further considerations arise when planning experiments on the basis of animal models of CKD-associated CVD, both general and specific to the research question. The generalizability of the model, given the age, sex, species and strain of the animals included in the experiment, is fundamental to the conclusions being drawn. For example although experiments are typically conducted in mice at age 8–12 weeks of age, older animals [84] are likely to better reflect the pathophysiology observed in human CKD, a disease of middle and old age [85]. Similarly, the sex of animals has been shown to influence the phenotype of models across a wide range of organ systems [86].

Species and strain are also important. Although mouse models are becoming more popular than rats because of the ability for genetic manipulation [16] and the lower cost, many of the CKD models rely on surgical procedures and CV measurements that are technically less challenging in larger animals such as rats. Furthermore, several strains of mice seem uniquely resistant to developing disease phenotype in several of the models described above (Table 1). In addition, rats used in experiments are typically outbred; thus, standardized nomenclature describing each strain, for example, 'Wistar', may be misleading as there can be a great deal of genetic heterogeneity within these broad strain descriptions [87].

Focusing on models of CKD-associated CVD, it is important to ensure that key CV findings are not related to the method of inducing kidney injury in the animal. Hence, any proposed pathway or intervention should be examined in at least two differing models of CKD, for example, one surgical and nephrotoxic model, and ideally across both rats and mice. Similarly, the heterogeneity of human CKD must be recognized, again implying any important findings are replicated across surgical, toxic and immunologically induced models of renal injury. Finally, the complexity of some of the procedures, particularly those related to CV phenotyping, means that only teams with extensive experience of performing this type of work can achieve high levels of reproducibility and minimize adverse events for experimental animals. To ensure compliance with the 'three R's', investigators without appropriate skills should ensure that they collaborate with those who do.

Conclusion

Although advances in technology may mean that this approach becomes redundant, animal models will likely continue to be a useful method to describe pathophysiology and test potential therapies in CKD-related CVD for decades to come. Thoughtful design of experiments, replication using different models and in-depth phenotyping using a wide range of techniques mean that we can maximize the benefits from these studies and, over the longer term, produce real benefits for patients.

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Conflicts of interest

There are no conflicts of interest.

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