

Animal models of atherosclerosis (Review)

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Abstract. Atherosclerosis is a significant cause of morbidity and mortality globally. Many animal models have been developed to study atherosclerosis, and permit experimental conditions, diet and environmental risk factors to be carefully controlled. Pathophysiological changes can be produced using genetic or pharmacological means to study the harmful consequences of different interventions. Experiments using such models have elucidated its molecular and pathophysiological mechanisms, and provided platforms for pharmacological development. Different models have their own advantages and disadvantages, and can be used to answer different research questions. In the present review article, different species of atherosclerosis models are outlined, with discussions on the practicality of their use for experimentation.

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1. Introduction

Atherosclerosis is an autoimmune condition characterized by the development of complex atherosclerotic plaques, leading to hardening and narrowing of the arterial lumen. Chronic exposure

to cardiovascular risk factors, such as hyperlipidemia, hypertension, smoking, male gender and diabetes, can increase the rate and severity of atherosclerosis. Among the different risk factors, increased plasma low-density lipoprotein (LDL) level has been identified as the most significant, which alone is sufficient to produce atherosclerosis in monogenetic hyperlipidemia disorders, such as familial hypercholesterolemia (1). In individuals with normal LDL levels, other factors are responsible for the development and progression of atherosclerosis (2,3). However, these risk factors are rather insignificant in individuals with low LDL levels, who are unlikely to develop atherosclerosis irrespective of the presence of additional risk factors (4).

Excess LDL in plasma accumulates in the sub-endothelial space of the arterial wall, undergoing oxidation to become oxidized LDL (oxLDL). This in turn, triggers an inflammatory response, thereby inducing the expression of a number of different molecules, such as vascular cell adhesion molecule-1, E-selectin and P-selectin in the endothelium (5). This response provides the necessary conditions for chemotaxis, where blood cells are recruited into the injured arterial wall (6). Monocytes are the most prominent cell type involved (7). After entry into the tunica intima, monocytes undergo differentiation into macrophages, which take up oxLDL to become foam cells (8). Foam cells function as antigen-presenting cells, and activate circulating monocytes and T-cells (9). They also secrete mediators to further perpetuate inflammation, and stimulate the migration of smooth muscle cells from the tunica media into sub-endothelial space (10). Mediated by platelet-derived growth factor, the smooth muscle cells exhibit abnormally high proliferation rates and secrete extracellular matrix proteins that contribute to fibrous cap formation (11). The fibrous cap protects the core of the plaque from circulating blood cells, especially platelets responsible for the thrombosis associated with rupture plaques. This maladaptive non-resolving inflammation is the driving force of atherosclerotic plaque development (12). SMCs from different regions of the microvasculature have different developmental origins (13), which can contribute to site-specific atherosclerotic responses (14).

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During plaque evolution, macrophages proliferate, undergo apoptosis and efferocytosis (15,16). Apoptotic cells may be removed, leading to lesion size reduction, or may accumulate and be subjected to secondary necrosis, producing the necrotic core characteristic of advanced plaques. Accumulation of apoptotic bodies may enhance the plaque instability by triggering inflammation. While foam cells are the most abundant leukocytes within the atherosclerotic lesions, other cell types, including neutrophils, mast cells, T-lymphocytes and B-lymphocytes are also involved in atherogenesis (17,18). Although these cells contribute little in mass to the lesions, they can secrete different signalling proteins that regulate other cells or components within the plaques (19-21).

Plaque rupture is responsible for the adverse clinical consequences of ischaemia in cerebrovascular accidents, myocardial infarction and heart failure, producing significant morbidity and mortality in affected patients. In advanced stages of atherosclerosis, rupture of vulnerable plaques exposes their thrombogenic compounds, producing luminal thrombosis. Destabilization of plaques into a vulnerable state is in part mediated by macrophage-derived proteases, such as metalloproteases; however, the precise mechanisms remain incompletely characterized (22).

2. Animal models of atherosclerosis

In 1908, Ignatowski provided the first experimental demonstrations that atherosclerosis can be induced in laboratory animals. He fed rabbits a protein-rich diet (mainly meat, milk, and egg yolk), which led to the formation of atherosclerotic lesions in the aortic wall. Since then, a number of species, such as rabbits, mice, rats, guinea pigs, hamsters, birds, dogs and non-human primates, have been developed. Despite differences between the animal models, a common finding is the necessary condition of hypercholesterolaemia in plaque development. Animal models have been extensively used for the study of human cardiovascular diseases (23-41). In the present review, we review rabbit, porcine and non-primate models of atherosclerosis, together with their advantages and disadvantages (Table I).

Rabbit. Rabbit is the first animal model developed for atherosclerosis research, leading to the identification of the crucial role of elevated plasma cholesterol in atherogenesis. It served as the mainstay of pre-clinical model until genetically modified mouse models became widely available.

New Zealand White (NZW) strain. The most common strain is the NZW, in which the roles of lipoproteins of differing sizes on atherosclerosis were examined. For example, atherosclerosis was unexpectedly inhibited in an alloxan-induced diabetes rabbit model, explicitly by the accumulation of large triglyceride-rich lipoprotein (>75 nm diameter), to which the vascular wall has limited permeability (42,43). However, NZW rabbits show high biological variability with respect to individual responsiveness to dietary cholesterol and the lesion morphology varies significantly depending on the cholesterol content of the diet (44). This strain is not prone to atherosclerotic risk due to its low plasma cholesterol level of 50 mg/dl when exposed to standard diet. The induction of

vascular lesion in NZW rabbits generally requires feeding of a high cholesterol diet (from 0.2 to 2% cholesterol) which increases plasma cholesterol level by ≤ 8 -fold and leads to the formation of foam cells-enriched fatty streaks in several vascular regions, especially the aortic arch and the thoracic aorta (45). For complex atherosclerotic plaques with lipid core surrounded by smooth muscle cells to develop, a long period of cholesterol feeding, from six months to several years, is required. The disadvantage of this diet is its hepatic toxicity, which increases mortality.

Genetically modified rabbits. Due to the noxious side effects of the high-fat diet, genetically modified rabbits have been developed to produce spontaneous atherosclerotic lesions. For example, Watanabe hereditary hypercholesterolemic rabbit (WHHL), a LDLR-deficient model, was used by Buja *et al.*, who identified LDL as the lipoprotein underlying human familial hypercholesterolemia (46). The advantage of this WHHL model is that the morphology of lesions and lipid metabolism are largely similar to those observed in humans. When WHHL rabbit is fed with 1% cholesterol for 12 months, the atherosclerotic plaques resemble those seen in homozygous familial hypercholesterolemia patients (46,47), with areas of necrosis, cholesterol clefts and calcification (48), with foam cells originating from smooth muscle cells. Furthermore, WHHL rabbits share the same gender predisposition patterns, with males being more prone to coronary atherosclerosis (49).

Advantages and disadvantages of rabbit models. Rabbits share the same advantages with mice with their small size and hence ease in maintenance, high availability and low economical cost. They are frequently preferred because of similar lipoprotein metabolism to humans. With the expression of CETP, the predominant lipoprotein in rabbit is LDL (45). Rabbits are sensitive to dietary cholesterol overload, demonstrating subsequent hyperlipidemia without the need of the toxic high cholesterol diet. Since rabbits are larger than mice, catheter-based procedure and non-invasive imaging can be used for experimental interrogation. Some important differences are that the frequent sites of atherosclerotic lesions in rabbits are the aortic arch and descending thoracic aorta, whereas those in humans are the coronary arteries and the abdominal aorta (45). Nevertheless, application of an ameroid constrictor to induce arterial stenosis in rabbit coronary arteries led to intimal proliferation together with eccentric narrowing 4 weeks later (Fig. 1) (30).

Porcine. The different porcine models can broadly be divided into wild-type or genetically modified models.

Wild-type pig models. Rapacz pig is a wild-type model with a natural mutation in *ApoB* and *LDLR* genes, which were produced by selective breeding of pigs with high cholesterol by Davis *et al.* (50). Within 2-4 years on a normal diet, these pigs developed increased hypercholesterolemia, with LDL as the main circulating lipoprotein, associated with the development of coronary atherosclerosis. A mini-pig model bearing the same gene mutation but with lower cost, the hypercholesterolemia Bretoncelles Meishan (FBM) pigs, was subsequently made available (51). The diabetic

Table I. Models of atherosclerosis with their advantages and disadvantages.

Animal experimental models

Rabbits
Advantages
Similar lipid metabolism with human
Similar morphology of lesion development
Low cost for maintenance
High availability
Larger artery allow clinical evaluation: Ultrasound and MRI can be applied to determine plaque composition and its vulnerability
Low cost of maintenance due to its small size
Disadvantages
Not always responsive to dietary cholesterol
Different cardiovascular physiology with human: HDL as the predominant plasma lipoprotein, absence of Apo AII, low hepatic lipase activity
Low hepatic lipase activity leads to hepatotoxicity following prolonged cholesterol feeding
Plaque lesion dissimilar with human: foam cells with more fatty streak and macrophage rich, advanced lesion (e.g., Fibrosis and haemorrhage and ulceration) are not seen
Different predilection site: Atherosclerotic plaque preferentially deposited in aorta, iliac arteries
Porcine
Advantages
Similar haemodynamics and pathogenesis to humans: Lesion location, morphology and content
Similar heart size and cardiovascular anatomy
Similar lipid metabolism, except for Apo II deficiency in porcine
Highly defined genotypes for genetic manipulation
Minipig version offer option with lower cost
Unlike mouse and rabbit, it can spontaneously develop atherosclerosis with an accelerated rate when fed with atherogenic diet
Easier to carry out imaging, e.g., Ultrasound, CT and MRI compared to smaller species
Disadvantages
Toxic diet needed for induction of atherosclerosis
Large in size, which limits its practical use
Non-human primates
Advantages
Similar cardiac anatomy: Same predilection site for atherosclerosis
Similar cardiac physiology: Comparable lipid metabolism and advanced atherosclerotic lesion found
Closest phylogenetic relationship with human
Highest resemblance to human atherosclerotic clinical condition
Susceptible to spontaneous atherosclerosis
Similar omnivorous diet
Disadvantages
High cost for purchase and maintenance
Long lifespan and hence long period of time needed for induction of atherosclerosis
Significant ethical concern
Large size with difficulty of management
Low availability

hypercholesterolemic wild-type pig can provide a humanoid model for investigations. An example is the type I diabetes model produced by intravenous streptozotocin injection, which has been used to destroy >80% of pancreatic β -cells in Yorkshire pigs (52). Its combination with a high cholesterol diet

used to induce hypercholesterolemia increased atherosclerotic risk by 2-fold, compared with hypercholesterolemia alone. Diabetic Yorkshire pigs demonstrated an accelerated rate of atherosclerotic lesion development in the aorta, coronary and femoral arteries (53). The lesions developed exhibit human-like

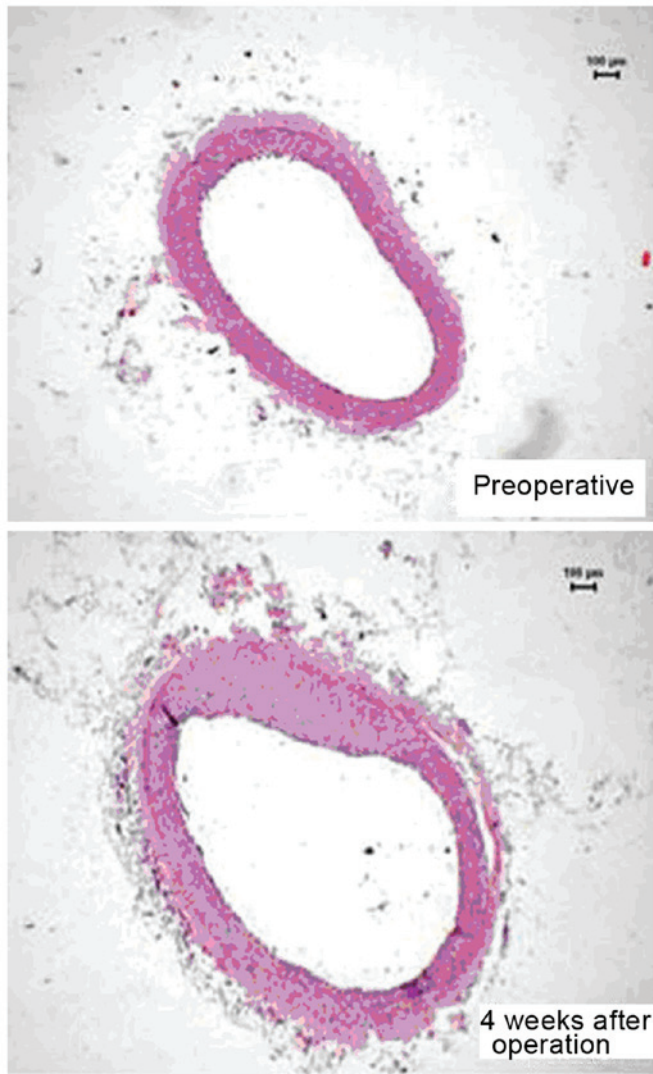


Figure 1. A cross-sectional image of a coronary artery in a rabbit model before (top) and 4 weeks after application of an ameroid constrictor to induce arterial stenosis, showing clear evidence of intimal proliferation with eccentric narrowing (bottom). Reproduced from (22) with permission.

features of advanced plaques, including necrotic cores, fibrous caps, calcification, medial thinning and intraplaque haemorrhages.

Genetically modified porcine models. In genetic mini-pig models, atherosclerosis can be induced without the use of cholic acid, thereby avoiding its toxic inflammatory side effects (54). An example of a model system is the Yucatan mini-pig with liver-specific expression of D374Y, a gain-of-function mutation in human protein convertase PCSK9, which consistently downregulated LDL receptor levels and increased LDL concentrations following a high-fat diet. This led to a severe form of autosomal dominant hypercholesterolemia (55,56), successfully inducing atherosclerosis in thoracic, abdominal, ilio-femoral and coronary arteries at 1 year of age. This model is applicable for validating equipment designed for human use, such as clinical scanners and intravascular devices. Another transgenic model is the LDLR knockout Yucatan mini-pigs, which demonstrated similar hypercholesterolemia and progression of atherosclerotic development. A high-fat

diet led to the development of atherosclerotic lesions at 6-11 months (57).

Advantages and disadvantages of porcine models. The use of porcine models has the advantage of bearing close resemblance of cardiac anatomy and physiology to the human counterpart. LDL, as in humans, is the major circulating lipoprotein in plasma, except for apolipoprotein II deficiency in pigs. Another advantage is the highly defined genotypes, which enable the development of porcine models with multiple genetic alterations. The emergence of site-specific nucleases, including the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, was a breakthrough that allowed multiple genes to be targeted at the same time by the expression of multiple sgRNAs together with the Cas9 nuclease. The CRISPR/Cas9 system allows pronuclear injection protocols with a high success rate (58), and only one animal cloning round is needed, reducing the time needed for breeding and the cost of production.

As with humans, pigs are susceptible to diet-induced hypercholesterolemia, but they require high dietary cholesterol, typically combined with cholic acid to block the conversion from cholesterol to bile (59,60). The atherosclerotic lesions usually do not progress beyond the foam cell stage and the duration of atheroma formation is longer than that observed in mice. Combining the use of an atherogenic diet with artificial vascular injury is one of the methods to accelerate atherosclerosis development in pigs. First, normal pigs are fed with a high cholesterol diet and percutaneous intramural injection of cholesteryl esters and human oxLDL (61,62). Two weeks later, vascular injury is produced by methods such as guidewire-induced injury, endovascular balloon inflation and partial vessel ligation (63). This method produces rapid atherosclerotic plaque development, thereby reducing the duration and cost of the experimental studies. The histopathology of atherosclerotic lesions are similar to humans, including their location and content (57). This model therefore provides a platform for the investigation of the disease complications, including plaque rupture, ischemic reperfusion injury, arterial thrombosis and restenosis after angioplasty (64-66), and explorations for therapeutic interventions such as drug-eluting stents (66).

The use of the porcine model as an *in vivo* validation of imaging tools is valuable, in contrast to the post mortem specimen and *ex vivo* model, which failed to produce satisfactory validation. Post-mortem specimens cannot imitate the dynamic cell components in atherosclerotic plaques, whereas *ex vivo* models do not demonstrate the pulsatile flow normally observed in elastic arteries, moving coronary arteries on a beating heart, irregular heart rhythms and the moving tissues surrounding the vessels. Initial validation is crucial for the development of intravascular imaging technique to guide therapy in symptomatic patients (67,68). With the authentic human-like dimension and morphology of coronary arteries, pigs provide an ideal platform for the insertion of intravascular imaging tools, such as intravascular ultrasound (69) and near-infrared spectroscopy (70). Real-time imaging of tissues and cells in evolving atherosclerotic plaques is also possible.

Due to lack of sensitive and reliable biomarkers for monitoring disease progression, imaging tools are important in

monitoring plaque evolution and the efficacy of therapeutic treatment. For non-invasive imaging with PET-CT, MRI or CT, high resolution imaging of large arteries in smaller animals is possible using dedicated and modified scan protocols. Porcine models are useful to establish the relationship between plaque size and system resolution, with similar extents of motion and artefacts compared to humans. The protocol of scan parameter can be used subsequently without the need of modification.

Another valuable use of the porcine model is drug development. In view of the close phylogenetic relationship and relevant atherosclerotic pathology observed in porcine systems (71,72), drug testing is predictive for efficacy of drugs in humans compared to the mouse model. It can be used to guide the decision on endpoint of drug efficacy in clinical trials. Currently, there is no standard imaging endpoint that is capable of detecting all beneficial effects of pharmacological intervention (73,74). Some drugs target lipid content reduction, which is potentially measurable using near-infrared spectroscopy (70). By contrast, other drugs that aim to reduce inflammation can be assessed by 18 fluorodeoxyglucose PET-CT techniques (75). Porcine models offer a test platform where both pathological examinations of atherosclerosis and evaluation with clinical imaging end point can be performed concurrently (71).

Nonetheless, the large size of pigs limits their widespread use. Recently, genetically engineered mini-pigs (76,77) in which hyperlipidemia and consequently atherosclerosis were successfully induced, became available; they are cheaper to maintain compared to full-sized pigs. A close examination of its pathophysiological mechanisms revealed similarities with human atherosclerosis, as in the full-sized pigs, that are not observed in mouse models.

Non-human primate models. Non-human primate models bear closest similarities to humans compared to other species, in terms of phylogeny with 98% genetic material being identical.

Rhesus and cynomolgus monkeys. Complex atherosclerotic lesions in coronary arteries of Rhesus monkeys were successfully induced using a high fat, high cholesterol diet (57). The lesions demonstrated intimal thickening and increased density of vasa vasorum in the tunica media (78), which are features also observed in humans. The identification of regression of coronary atherosclerosis upon reversion to a low-fat diet was first established using this model system (79). This was associated with a lower cholesterol content within the lesions and a decrease in the number of foam cells as well as their lipid content. Cynomolgus monkeys have been used because of their higher sensitivity to a high-fat diet. When these animals were fed with 12.5% coconut oil and 12.5% of butter fat (50,80), their plasma cholesterol level was twice as high as those of the Rhesus monkeys, associated with a higher number of lipid-loaded monocytes in the blood and skin xanthomata, as well as faster disease progression.

Advantages and disadvantages of primate models. The major advantage of using primates is that they have very similar cardiac anatomy and physiology compared to humans. Abnormal cardiovascular physiology in terms of lesion morphology,

plaque vulnerability and development of spontaneous luminal thrombosis are observed in both species (51,80-82). Primates bear similar susceptibility to atherosclerosis, with younger-aged animals being reasonably resistant to development of atherosclerosis, but have an increased risk of becoming susceptible with increasing age (83). Gender difference in the susceptibility of atherosclerosis have been demonstrated in these primate models, with a male preponderance to development of atherosclerosis following the introduction of a high-fat diet (84). High cholesterol diet greatly accelerated the development of atherosclerosis and frequently induced fatal MI in these primates (85). Conversely, disease regression upon low fat feeding was also evident, in keeping with clinical findings (86,87). Finally, associations between psychosocial factors and atherosclerosis have similarly been established for these primate models (88). Taken together, these factors lead to a greater applicability of experimental data on the clinical scenario.

Despite bearing close resemblances to humans, primate models are less popular than the other types of model due to its difficulty in maintenance due to their large size, high cost, limited availability and the special facilities required for their accommodation. Secondly, a considerable length of time is needed to induce significant atherosclerosis. Thirdly, the ethical concern of experimenting with human-like primates limits their widespread use. Nevertheless, non-human primates are ideal for the development of reliable biomarker tools for risk stratification and monitoring of the effects of pharmacological interventions on disease progression.

3. Concluding remarks

Animal models have been extensively used to study the pathophysiology of cardiovascular disorders (89-106). There is no one single ideal animal model for all the diseases (107,108). The general criteria for an appropriate animal model includes the size, docility, ease of breeding and housing, known genetic profile, analogies with humans and the cost associated. A smaller animal model, such as mouse and rabbit, generally provide valuable information on etiology and pathophysiology of atherosclerosis. Understanding of the risk factors and the natural history of atherosclerosis offer insights on disease prevention. On the other hand, larger animal models, such as porcine and non-human primates, are more reliable homologues with human disease. The advanced lesions developed share similar histological features with humans, from initial content of fatty streak to final advanced lesion of ulceration and thrombus formation. Their use is therefore more valuable for the development of disease management, such as analysing the utility imaging methods and assessing the efficacy of pharmacological intervention.

With the advancement in genetic technology, the development of mini-pigs is a favourable trade-off between human-like physiology compared to non-human primate; and ease of handling compared with small animal, with high resemblance to human cardiac anatomy, physiology, lipid metabolism and atherosclerotic pathophysiology. IT is expected to act as an important *in vivo* model, for developing sensitive biomarkers and validated imaging tools to predict plaque rupture, as the most important clinical event that cost life in atherosclerosis.

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