Principles and Applications of Rabbit Models for Atherosclerosis Research

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Rabbits are one of the most used experimental animals for biomedical research, particularly as a bio-reactor for the production of antibodies. However, many unique features of the rabbit have also made it as an excellent species for examining a number of aspects of human diseases such as atherosclerosis. Rabbits are phylogenetically closer to humans than rodents, in addition to their relatively proper size, tame disposition, and ease of use and maintenance in the laboratory facility. Due to their short life spans, short gestation periods, high numbers of progeny, low cost (compared with other large animals) and availability of genomics and proteomics, rabbits usually serve to bridge the gap between smaller rodents (mice and rats) and larger animals, such as dogs, pigs and monkeys, and play an important role in many translational research activities such as pre-clinical testing of drugs and diagnostic methods for patients. The principle of using rabbits rather than other animals as an experimental model is very simple: rabbits should be used for research, such as translational research, that is difficult to accomplish with other species. Recently, rabbit genome sequencing and transcriptomic profiling of atherosclerosis have been successfully completed, which has paved a new way for researchers to use this model in the future. In this review, we provide an overview of the recent progress using rabbits with specific reference to their usefulness for studying human atherosclerosis.

Key words: Animal models, Hypercholesterolemia, Atherosclerosis, Rabbits, Transgenic

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that seen in humans and proposed a causal role of cholesterol in atherosclerosis\textsuperscript{5)}. Their studies established the basis of atherosclerosis theory that in both humans and experimental animals, dietary cholesterol can induce atherosclerosis. Rabbit models were widely used and disclosed most of the pathophysiological significance between human atherosclerosis and lipid metabolism such as the discovery of LDL receptors and development of statins, the most prescribed lipid-lowering drugs in the world\textsuperscript{7).} Compared with mice, laboratory rabbits are sensitive to a high cholesterol diet and rapidly develop hypercholesterolemia\textsuperscript{7).} Therefore, it is much easier to induce hypercholesterolemia in rabbits than in other animals by feeding a high cholesterol diet. Dietary cholesterol can range from 0.3\% to 1\% depending on the research purposes. We recommend the use of 0.3\% to 0.5\% cholesterol in the diet for most experiments because it produces less health problems such as liver toxicity. If cholesterol content is too high (>1\%), rabbits develop massive hypercholesterolemia (plasma cholesterol levels > 1200 mg/dl), which is never observed in humans. Plasma cholesterol levels that are too high often lead to systemic lipid accumulation and liver dysfunction in rabbits, which is often criticized as “not physiological” by many researchers. Therefore, it is not recommended to feed rabbits with a diet containing cholesterol higher than 1\% for more than 4 weeks. In addition to cholesterol, oil, such as soybean or corn oil, is essential in the diet. This is because oil in the diet helps in absorption of intestinal cholesterol. There are many commercially available dietary oils that may be used for rabbit experiments. However, coconut oil, as an exception, is rich in saturated fatty acids, unlike other vegetable dietary oils that are rich in polyunsaturated fatty acids. Coconut oil can be used for insulin resistance studies\textsuperscript{18, 19).} It should be noted that cholesterol diets without the addition of oil lead to mobilization of internal fat tissue for absorption of

### Table 1. Comparison of lipid and lipoprotein metabolism features

| Feature                                      | Human | Rabbit | Mouse |
|----------------------------------------------|-------|--------|-------|
| Major plasma lipoproteins                   | LDL   | LDL    | HDL   |
| CETP                                         | Abundant | Abundant | None |
| Hepatic apoB mRNA editing apoB-48            | Chylomicrons | Can be bound to apo(a) | VLDLs/LDLs and Chylomicrons |
| apoB-100                                     | Can be bound to apo(a) | Heterogeneous | Cannot be bound to apo(a) |
| HDL                                          | Heterogeneous | Absent | Homogeneous |
| apoAll                                       | Dimer | Down-regulated | Monomer |
| Hepatic LDL receptor activity                | High, liver-bound | Low, liver-bound | High, 70\% in circulation |
| Hepatic lipase                               | Mainly from hepatic synthesis | Mainly from hepatic synthesis | Mainly from dietary origin |
| Cholesterol pool                             | Low | Low | Low |
| Excretion of bile acid                       | Sensitive | Sensitive | High |
| Response to a high cholesterol diet          |       |       |       |

#### Methods to Induce Atherosclerosis in Rabbits

In general, aortic atherosclerotic lesions in rabbits can be easily induced by feeding a high cholesterol diet alone or in combination with arterial balloon injury. Compared with other animals, especially rodents, laboratory rabbits are sensitive to a high cholesterol diet and rapidly develop hypercholesterolemia\textsuperscript{7).} Therefore, it is much easier to induce hypercholesterolemia in rabbits than in other animals by feeding a high cholesterol diet. Dietary cholesterol can range from 0.3\% to 1\% depending on the research purposes. We recommend the use of 0.3\% to 0.5\% cholesterol in the diet for most experiments because it produces less health problems such as liver toxicity. If cholesterol content is too high (>1\%), rabbits develop massive hypercholesterolemia (plasma cholesterol levels > 1200 mg/dl), which is never observed in humans. Plasma cholesterol levels that are too high often lead to systemic lipid accumulation and liver dysfunction in rabbits, which is often criticized as “not physiological” by many researchers. Therefore, it is not recommended to feed rabbits with a diet containing cholesterol higher than 1\% for more than 4 weeks. In addition to cholesterol, oil, such as soybean or corn oil, is essential in the diet. This is because oil in the diet helps in absorption of intestinal cholesterol. There are many commercially available dietary oils that may be used for rabbit experiments. However, coconut oil, as an exception, is rich in saturated fatty acids, unlike other vegetable dietary oils that are rich in polyunsaturated fatty acids. Coconut oil can be used for insulin resistance studies\textsuperscript{18, 19).} It should be noted that cholesterol diets without the addition of oil lead to mobilization of internal fat tissue for absorption of
RT-PCR methods. If these analyses are required, these samples should be immediately frozen in nitrogen liquid and stocked at \(-80^\circ C\).

Gross analysis usually requires Sudan IV staining to visualize atherosclerotic lesions followed by image analysis to calculate the lesion percentage of each aortic segment \(23\). Based on the gross observations, one can decide which parts to select to make paraffin-embedded sections for microscopic observations. Usually, the whole aortic arch and thoracic aorta will be cut into 10-12 segments (1-mm thick) for paraffin embedding \(23\). Serial sections can be routinely used for hematoxylin-eosin (HE) staining and elastic van Gieson (EVG). HE staining is essential to evaluate the quality of the lesions such as cellular and extracellular matrix component features and plaque vulnerability. To quantify the intimal lesion area on the sections, EVG staining is useful because it can visualize the internal elastic lamina (IEL), making it easy to measure intimal lesions and medial lesions separately. Paraffin sections can be used for direct immunohistochemical staining in most cases using most commercial antibodies. Routinely, it is essential to evaluate macrophages and smooth muscle cells using RAM-1 and smooth muscle \(\alpha\)-actin antibodies (Fig. 1).

If analysis of protein and mRNA expression is required, a piece of fresh tissue proceeded in liquid nitrogen at the time of aorta collection should be collected. In hypercholesterolemic rabbits, iliac arteries, carotid arteries and subclavian arteries seldom exhibit atherosclerosis; however, coronary lesions of rabbits can

**Methods to Analyze Atherosclerosis**

The rabbit aorta is mainly used for the analysis of atherosclerosis because it is easy to isolate and both gross and microscopic evaluation of aortic lesion size and quality can be made. The lesions usually start to form from the aortic arch followed by the intercostal orifice area and thoracic aorta. Abdominal aortic lesions are less frequently seen, which is different from human atherosclerosis. As the aortic lesions are large, it is also possible to collect a part of the lesions to analyze protein and mRNA expression using Western blotting and

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**Fig. 1.** Representative aortic lesions in cholesterol-fed rabbits. A. Gross lesions of the aorta stained by Sudan IV (visualized as red area). B. Representative micrographs of early stage lesions (fatty streaks). C. A typical fibrous plaque with a lipid core covered by a fibrous cap. Serial paraffin sections of the aortic arch were stained with hematoxylin and eosin (HE) and elastic van Gieson (EVG), or immunohistochemically stained with monoclonal antibodies (mAbs) against either macrophages (M\(\phi\)) or \(\alpha\)-smooth muscle actin for smooth muscle cells (SMC).
ally expressed in the liver or macrophages to study their functions (Table 2). To date, more than 20 genes have been introduced into Tg rabbits, and have provided considerable insight into the molecular mechanisms associated with their functions in lipoprotein metabolism and atherosclerosis²⁵).

Knock-Out Rabbits

For a long time, generation of knock-out rabbits has been a dream for researchers because rabbit embryonic stem (ES) cells are not available and genomic information for rabbits is lacking. Rabbit genome sequencing was initially completed by a European group²⁶), and two years later, we along with Chinese and US groups, successfully sequenced three rabbit genomes (New Zealand White, Japanese White and Watanabe heritable hyperlipidemic rabbits) and completed transcriptomic profiling of atherosclerotic lesions and the liver for these rabbits²⁷). These genomic and RNA seq data will provide many insights into understanding the pathogenesis of atherosclerosis in rabbits. Another advancement in this field is the emergence of novel gene-editing technologies (ZFN, TALENs, CRISPR-Cas9) in succession, which has made it possible to make KO rabbits just as KO mice. The first KO rabbits were

Table 2. Transgenic rabbits for the study of human cardiovascular diseases

| Genes                  | Expressing cells | Major phenotypes                        | References |
|------------------------|------------------|-----------------------------------------|------------|
| Apolipoproteins:       |                  |                                         |            |
| Apo(a)                 | Liver            | Atherogenic                             |            |
| ApoA-I                 | Liver            | Athero-protective                       |            |
| ApoA-II                | Liver            | Athero-protective                       |            |
| ApoB-100               | Liver            | LDL †, HDL ‡                           |            |
| ApoCⅢ                  | Liver            | VLDL †                                 |            |
| ApoE2                  | Liver            | Atherogenic                             |            |
| ApoE3                  | Liver            | Atherogenic                             |            |
| Enzymes:               |                  |                                         |            |
| Hepatic lipase         | Liver            | Athero-protective                       |            |
| apoB mRNA editing protein | Liver          | LDL ‡                                  |            |
| LCAT                   | Liver            | Athero-protective                       |            |
| Lipoprotein lipase     | Universal        | Athero-protective                       |            |
| PLTP                   | Universal        | Atherogenic                             |            |
| Endothelial lipase     | Liver            | Athero-protective                       |            |
| Vascular factors:      |                  |                                         |            |
| Lipoprotein lipase     | Macrophage       | Atherogenic                             |            |
| MMP-12                 | Macrophage       | Atherogenic                             |            |
| C-reactive protein     | Liver            | Thrombogenic                            |            |
| 15-lipoxygenase        | Macrophage       | Athero-protective                       |            |
| VEGF                   | Liver            | hemangiomas and impaired glomerular functions |            |

ND: not done. LCAT, lecithin: cholesterol acyltransferase; PLTP, phospholipid transfer protein; MMP, matrix metalloproteinase; VEGF, vascular endothelial cell growth factor.

be observed at the large branches such as the left and right coronary arteries. The method for rabbit coronary lesion analysis has been described in detail in the previous review⁷). Balloon injury of aortas or wire injury of iliac arteries in rabbits is sometimes used to produce intimal lesion models. However, these lesions induced by injuries are mainly composed of proliferating smooth muscle cells forming so-called “neo-intima”, which is different from fatty streaks induced by cholesterol diet feeding. However, balloon injury may also be performed in cholesterol-fed rabbits, which will accelerate the formation of the lesions²⁴).

Transgenic Rabbits

In addition to cholesterol-fed rabbits, transgenic (Tg) rabbits have also been used for the study of human cardiovascular disease and lipoprotein metabolism during the last two decades. However, in contrast to Tg mice, Tg rabbits were only used by a few institutions to study atherosclerosis. Elucidation of gene functions in terms of lipoprotein metabolism and atherosclerosis using Tg rabbits was essentially based on the premise that rabbits bear features more similar with those of humans such as metabolism and susceptibility to atherosclerosis (Table 1). Transgenes are usu-
sion regarding whether to use rabbits. The most critical point is whether experiments are designed to solve clinical problems such as for translational research.

In conclusion, rabbits are still a powerful model for the study of human atherosclerosis. With the advances in nuclease-editing technology, KO rabbits will provide a new means for translational research in this field. These new models will help develop novel therapeutics and diagnostics for cardiovascular disease in the future.

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Conflicts of Interest
None.

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