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The rabbit in atherosclerosis research

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Introduction

The rabbit was the first animal model used in atherosclerosis research (*Constantinides* 1965, *Jokinen et al.* 1985). In 1908, Ignatovsky induced atherosclerosis in rabbits by feeding them milk meat and eggs (*Glueck* 1975, *Jokinen et al.* 1985). In 1913, Anitschnikov obtained similar lesions by feeding rabbits pure cholesterol (*Glueck* 1975). These Russian experiments introduced the way of the metabolic concept of atherosclerosis, a disease of the arterial intima and media, characterized by deposition of cholesterol and fat due to a complex and dynamic interaction between plasma lipoproteins and cells of the arterial wall. Furthermore, they provided the first evidence for the induction of atherosclerosis in laboratory animals similar to that of man by dietary changes. In subsequent years, different animal species have been used to study development of atherosclerosis (*Vesselinovitch* 1975, *Mc Cauley & Bull* 1980, *Malinov* 1983, *Jokinen et al.* 1985, *Wójcicki et al.* 1985, *Betz* 1987, *Armstrong & Heistad* 1990, *Overturf & Loose-Mitchell* 1992). The rabbit, however, has been the most popular species in atherosclerosis research.

The aim of this paper is to give a review of the use of laboratory rabbit in atherosclerosis research, and to present the variants of the rabbit model including different study designs for cholesterol-fed rabbit, and to present methods for evaluation of experimental atherosclerosis.

Criteria for an ideal animal model of human atherosclerosis

The search for better animal models of human atherosclerosis led to formulation of several criteria for the ideal model (*Vesselinovitch* 1975, *Mc Cauley & Bull* 1980, *Jokinen et al.* 1985, *Stehbens* 1986, *betz* 1987). During the last 30 years the views on animal models and their role in the studies of atherosclerosis have changed. Therefore, some of the criteria lost their importance and some new criteria have been added.

In general, the criteria concern three topics: (1) the nature of experimental atherosclerosis, (2) biology of the chocen species, and (3) practical aspects in the relation to the experimental procedure.

The nature of experimental atherosclerosis. The purpose is to ensure the similarity between the experimentally induced disease in laboratory animals and that naturally occuring in man. Therefore, the experimental atherosclerosis should develop under conditions comparable to conditions existing in man *eg.* due to hyperlipidemia caused by the exogenous factor such as an atherogenic diet, not too different from the standard feed, or by the endogenous factor such as genetic disorders in lipid metabolism. The atherogenic factor should lead to the development of atherosclerotic lesions in arterial walls with a morphology and topography similar to those in man. The progression of the experimental process should lead to formation of advanced lesions ie. atherosclerotic plaques with macrophages, cholesterol crystals, lipid and possible necroses, often deep-seated and covered by fibrous cap. Some complications of the disease process such as aortic aneurysm, myocardial and cerebral infarctions, and gangrene of extremities should occassionally appear in the ideal animal model.

The biology of the chosen species. The purpose is to provide the well characterized biological material for the study in order to facilitate the interpretation of the results. Therefore, the chosen species should have

defined genetic characteristics, and the anatomy, biochemistry and function of the cardio-vascular system should be close to those of man. A thorough information on spontaneous atherosclerosis in the species is necessary.

The practical aspects of species selection. The purpose is to facilitate the realisation of the experiment. Therefore, the chosen species should be cheep, easy to acquire, handle and house, especially when large number of animals is needed. The animals should have the proper size to allow the experimental manipulation eg. angiography, historical examination of aorta and preferably also coronary arteries.

The rabbit fulfils both the practical criteria and the criteria concerning the biology of chosen species. Being one of the most popular laboratory animal the rabbit has well known genetics, physiology and pathology. The spontaneous atherosclerosis has been described (*Haust & More* 1965, *Schenk et al.* 1966a, 1966b) and the blood lipid profile has been investigated (*Fillos & Mann* 1956, *Roberts et al.* 1974, *Day* 1979). The experimental atherosclerosis due to cholesterol feeding, the socalled cholesterol atherosclerosis, has been characterised by light microscopy (*Constantinides* 1965, *Pollak* 1965, *Lee et al.* 1978) and both strain and age variations in response to cholesterol diet have been described (*Pollak* 1965, *Spagnoli et al.* 1991).

Critique and limitations of the rabbit in atherosclerosis research

The critique of the rabbit is addressed towards the nature of experimental atherosclerosis. The rabbit is herbivorous, while the human is omnivorous. It has physiologically low plasma cholesterol compared to man, and the cholesterol diet provokes an extreme increase in plasma cholesterol to levels never seen in man. The major carrier of the plasma cholesterol in cholesterol-fed rabbit is *beta*-very low density lipoprotein (VLDL) while in man the low density lipoprotein (LDL). The topography of lesions is different, since experimental atherosclerosis in the rabbit mainly developes in the aortic arch and thoracic aorta, and not in the abdominal aorta as in man. However, the most severe critique has been addressed towards the morphology of the experimental atherosclerotic lesions in rabbits fed high cholesterol (1–3 % w/w) and fat (4–10 % w/w) diet. These rabbits develop lesions consisting of lipid-laden macrophages, and furthermore deposits of lipid-ladden macrophages are seen in parenchymatous organs. The complications of atherosclerotic plaques such as pronounced calcification, ulceration, haemorrhage and thrombosis leading to luminal stenosis have been never seen. Therefore in fifties and sixties, some of the defenders of the rabbit in atherosclerosis research made efforts to produce morphologically "human-like" complicated lesions using following methods: (1) the lipogenic: exposure of rabbits to marked intermittent lipemia caused by feeding the atherogenic diet over a long period, (2) the injury: injury of arteries followed by slight lipemia due to feeding the atherogenic diet for a short period, (3) thrombogenic: use of hypertension and hypercoagulability to induce local or embolic mural thrombi in aortic wall with already existing atherosclerotic lesions caused by one of the methods mentioned above, and transformation of these changes into fibrofatty plaques (*Constantinides* 1965).

According to recent standards for animal studies, the morphological resemblance of experimental atherosclerotic lesions in animal to atherosclerotic lesions in man is of no value if there are no common points in the mechanisms of lesion formation (Armstrong & Heistad 1990). The etiology of the human-like lesions caused by the injury and thrombogenic methods has little to do with the etiology of human atherosclerosis. Therefore the injury method has not gained broad application in studies on the influence of dietary factors in atherosclerosis. This is also the reason for abandoning of the thrombogenic method, and this method would furthermore be difficult to accept considering the animal

welfare and ethics in work with laboratory animals. The induction of human-like lesions by the lipogenic method is in accordance with the lipid hypothesis on etiology of human disease. However, this method is more time consuming than present methods *eg.* the shortest induction time of human-like lesions was seven months (two periods of lipemia of two months each separated by 3 month period on standard diet), but cases with intermittent lipemia repeated up to two years are described (*Constantinides* 1965).

The rehabilitation of the rabbit

The rabbit withstood the critique and gained new respect for several reasons. First, other type of lesions than plaques consisting of lipid-laden macrophages have been induced in the cholesterol-fed rabbit by lowering of cholesterol doses and/or shortening the induction (*Armstrong & Heistad* 1990, *Overturf & Loose-Mitchell* 1992). Second, the behaviour of lesions in experimental hyperlipidemia in rabbits tends to parallel that of the lesions in other species (*Armstrong & Heistad* 1990). Third, the New Zealand White rabbit has been classified as a "LDL mammal" along with humans, on the basis that the sum of the VLDL, intermediate density lipoproteins (IDL), and LDL accounts for more than 50 % of the total plasma lipoproteins (*Overturf & Loose-Mitchell* 1992). Fourth, of great importance in the rehabilitation of the rabbit has been the development of genetic variants of New Zealand White strain which develop hyperlipidemia due to genetic disorders in lipid metabolism.

Present rabbit models in atherosclerosis research

The following rabbit models are at present the most used in atherosclerosis research: (1) cholesterol-fed rabbit, (2) Watanabe heritable hyperlipidemic (WHHL) rabbit, and (3) St. Thomas' Hospital rabbit.

Cholesterol-fed rabbit

This model is based on normolipidemic rabbit strains, of which the New Zealand White (NZW) is the most popular. The experimental atherosclerosis develops due to hyperlipidemia caused by doses of egzogen cholesterol usually lower than 0.5 % (w/w) in the diet *eg.* 0.1 % (*Spagnoli et al.* 1991), 0.3 % (*Zhu et al.* 1990), 0.3 g/rabbit/day (*Haarbo et al.* 1991). Low cholesterol doses lead to formation of fibrocellular, human-like lesions. However, the higher cholesterol doses like 1–2 % are still used (*Cooke et al.* 1992, *Thiery & Siedel* 1987, *Jezierski et al.* 1993).

The fact that the major carrier of plasma cholesterol in cholesterol-fed rabbit is the *beta*-VLDL is no longer considered a disadvantage but an advantage, as it has been linked to the condition in the human known as broad-*beta* disease (type II hyperlipidemia), where defective binding attributable to an abnormal apoE leads to an accumulation of *beta*-VLDL or remnant particles (*Overturf & Loose-Mitchell* 1992).

The cholesterol-fed rabbit is widely used to study the role of dietary factors on development of atherosclerosis and it is the classical model to study the lipoprotein metabolism. For example, use of this model revealed important aspects of HDL metabolism (*Badimon et al.* 1989, 1990). The relevance of cholesterol-fed rabbit model to studies of lipoprotein metabolism is supported by its response to cholesterol-lowering agents and this is the reason for the extensive use of this model for the screening of potentially important hypocholesterolemic agents. Limited use of cholesterol-fed rabbit to study the atherosclerosis regression has been reported (*Malinov* 1983, *St. Clair* 1983, *Zhu et al.* 1990).

In the classical study design with cholesterol-fed rabbit, all groups receive the same cholesterol dose during the whole experimental period, regardless of dietary-medicamental treatment (*Mortensen et al.* 1993). The hypercholesterolemia is not controlled deliberately, but the cholesterol dose can be changed during the study if plasma cholesterol increases unsatisfactory (either to high or to low). To prevent the development of too

strong hypercholesterolemia in cholesterol-fed rabbits another study design can be applied. The cholesterol doses for the reference group are current adjusted in order to maintain the mean plasma cholesterol concentration in this group at a certain aimed level. The other experimental groups receive the same cholesterol doses as the reference group (*Andersen et al.* 1993). Both designs give possibility to study the effect of treatment on experimental atherosclerosis due to changes in plasma lipids. To investigate the effect of treatment on experimental atherosclerosis due to mechanisms not mediated by a change of total plasma cholesterol another study design can be applied. All rabbits, regardless of treatment, are maintained at the same plasma cholesterol levels during the whole experiment. It is achieved by the current individual adjustment of cholesterol doses every fifth or seventh day. This study design has been used in drug trial (*Brattsand et al.* 1974) and to compare the atherogenicity of various fats (*Leth-Espensen et al.* 1988, *Mortensen et al.* 1992).

Watanabe Heritable Hyperlipidemic (WHHL) Rabbit

Homozygous WHHL rabbit

WHHL rabbit is a strain of NZW rabbit obtained by inbreeding from a single hyperlipidemic mutant discovered in 1973 by Watanabe (*Kondo & Watanabe* 1975, *Watanabe* 1980). Currently, it is the most important model of familial hypercholesterolemia (*Shiomi et al* 1987, *Havel et al.* 1989). The homozygous animals have reduced number of functional LDL receptors, they exhibit strong hypercholesterolemia and moderate hypertriglyceridemia, and spontaneously develop atherosclerosis at young age. Their atherosclerotic lesions are considered close approximation of the human lesions (Fig. 1, 2 c and d). The development and morphology of atherosclerotic lesions in homozygous WHHL rabbits is well characterized (*Buja et al.* 1983, *Rosenfeld et al.* 1987a, b, *Fischer Hansen et al.* 1994). The WHHL rabbit, the homozygous phenotype being difficult to breed, is generally maintained in outbred lines. This heterogenicity results in increased inter-animal variability in concentrations of plasma lipids and degree of atherosclerosis. This also leads to the differences in the genetic background of the WHHL rabbits from the various colonies. Therefore it has been suggested, that WHHL rabbits of different breedings cannot be regarded as genetically equivalent (*Overturf & Loose-Mitchell* 1992).

The homozygous WHHL rabbit has been used to study the development and progression of atherosclerosis (*Rosenfeld et al.* 1987a, b), and to examine the effect of dietary and medicamental intervention (*Rich et al.* 1989, *Clubb et al.* 1989, *Lichtenstein & Chobanian* 1990, *Carew et al.* 1987, *Nagano et al.* 1989, *Mao et al.* 1991).

Heterozygous WHHL rabbit

The heterozygous WHHL rabbit has received little attention, most likely because it develops minimal spontaneous atherosclerosis, which is not observable until the age of approximately two years (*Atkinson et al.* 1989, *Esper et al.* 1993a, b, *Fischer Hansen et al.* 1994) (Fig. 2 a and b). The development of atherosclerotic changes in younger heterozygous WHHL rabbits can be induced by cholesterol feeding as in other normolipidemic rabbits. The cholesterol-fed heterozygous WHHL rabbits develop the fibro-cellular atherosclerotic lesions, morphologically not too different from lesions seen in homozygous WHHL rabbits (*Atkinson et al.* 1989, *Mortensen et al.* 1993). One percent cholesterol in the diet with or without added fats causes in WHHL heterozygots the pronounced atherosclerosis and the lipid infiltration in different organs (Fig. 3). The latter may indicate that the lower cholesterol doses should be used to induce the experimental atherosclerosis without provoking the cholesterol overload in other tissues. It has been proposed (*Atkinson et al.* 1989) that the cholesterol-fed heterozygous WHHL

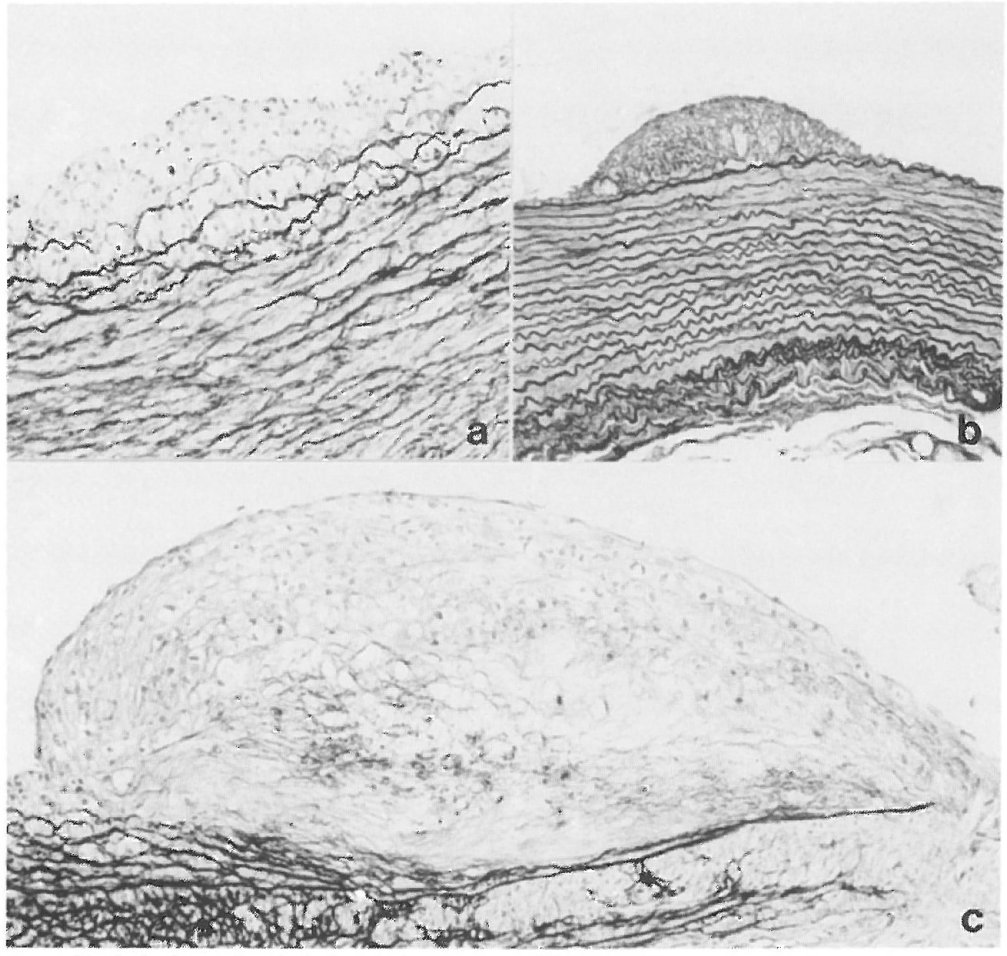

Fig. 1. Typical atherosclerotic lesions in homozygous WHHL rabbits. a: fatty streak, i.e. subendothelial band-like accumulation of foam cells. b: fibrous plaque, i.e. localized intimal thiskening with occasional foam cell. c: advanced lesion, i.e. accumulation of foam cells in fibrous stroma with formation of deep seated plaque.
(elastin van Gieson stains, a and c × 100, b × 40).

rabbit to greater extent simulates human population than the homozygous WHHL rabbit, since humans with the heterozygous familial hypercholesterolemia outnumber those with the homozygous form. The cholesterol-fed heterozygous WHHL rabbit has been used in our laboratory to study the effect of marine oils on experimental atherosclerosis (*Mortensen et al.* 1993).

St. Thomas' Hospital rabbit strain

This rabbit model has some characteristics similar to human familial combined hyperlipidemia. This genetically hyperlipidemic rabbit is characterized by elevated plasma levels of VLDL, intermediate density lipoproteins (IDL), and/or LDL which appear to be caused by an overproduction of the lipoproteins (*La Vile et al.* 1987, *Nordestgaard*

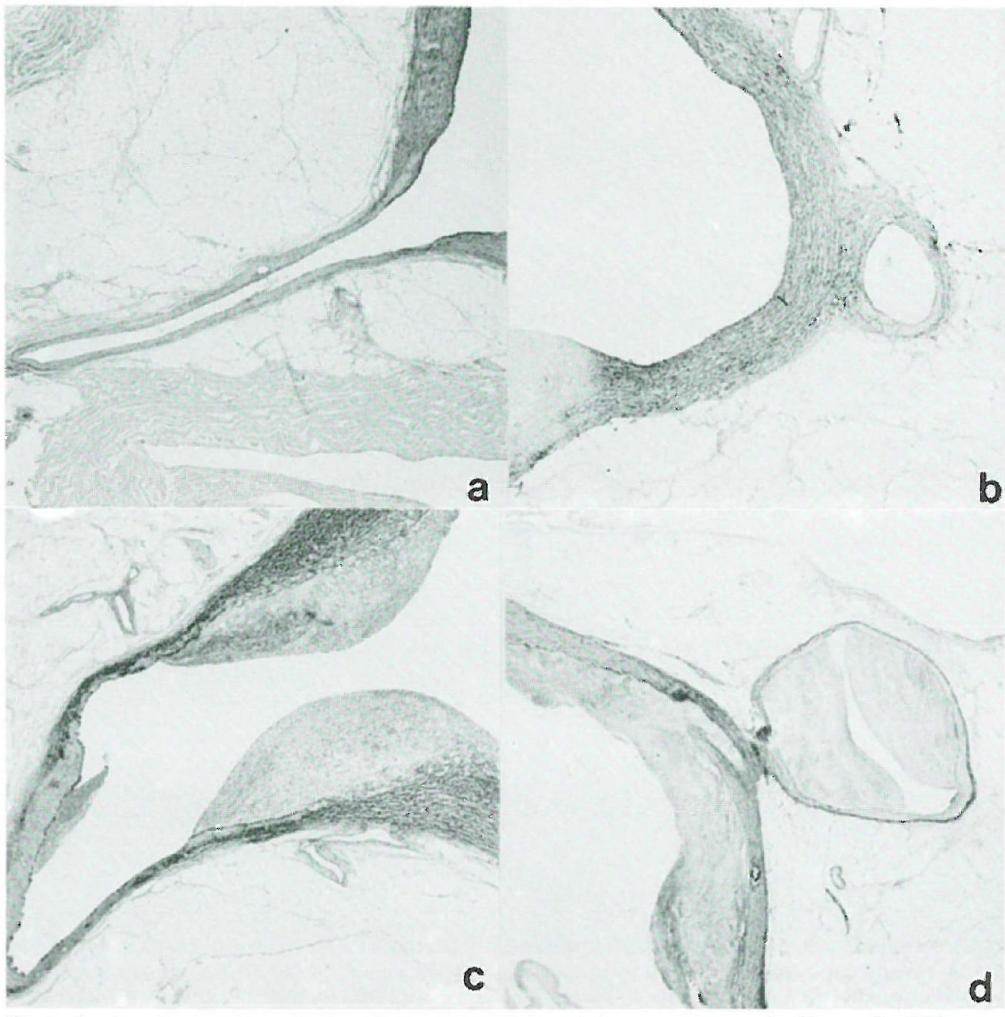

Fig. 2. Aortic orifices of the right (a and c) and the left (b and d) coronary artery in 19 months old heterozygous (a and b) and homozygous (c and d) rabbits. No lesions are observed in heterozygous animals whereas severe stenosing atherosclerosis is demonstrated in homozygous animals.
(elastin van Gieson stains, all × 40).

& Lewis 1990, *Nordestgaard et al.* 1992). This model has been used to study the relative atherogenecity of these particles.

Recently the great interest is given to the new strains of NZW rabbits characterized by hypo or hyperresposivenes to dietary cholesterol (*Armstrong & Heistad* 1990). It is expected that these strains may be helpful in studies on mechanisms regulating the plasma cholesterol levels (*Overturf & Loose-Mitchell* 1992).

Methods for evaluating of the experimental atherosclerosis

Atherosclerosis in the rabbit can be evaluated by macroscopic, biochemical and microscopic methods.

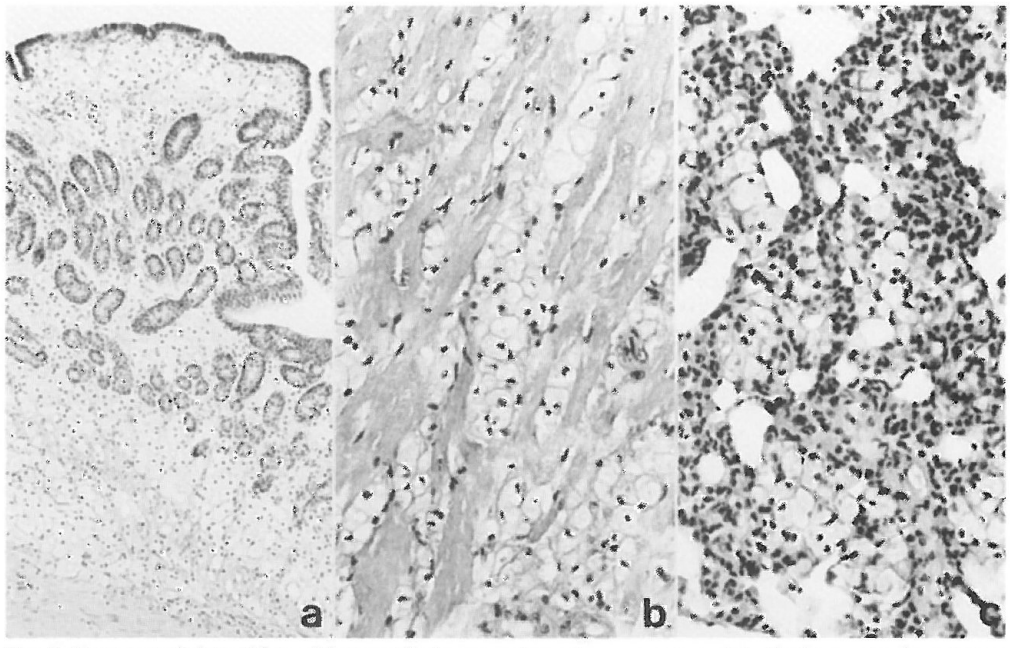

Fig. 3. Pronounced deposition of foam cells in parenchymatous organs. a: intestinal mucosa, b: myocardium, and c: lung.
(elastica van Gieson stains, all × 100).

Macroscopic methods like (1) naked eye evaluation using different grading systems (*Wu et al.* 1988, *Nordestgaard & Lewis* 1990) and (2) morphometry of lipid-positive areas done with a computer-assisted planimetry (*Atkinson et al.* 1989, *Clubb et al.* 1989, *Rich et al.* 1989), or by point-counting (*Fischer Hansen et al.* 1994) are performed on longitudinally opened aorta to measure the disease extent.

Biochemical determination of cholesterol content in aorta (*Nordestgaard & Lewis* 1990, *Mortensen et al.* 1992, *Fischer Hansen et al.* 1994) performed usually in the tissue of aortic intimal layer is regarded as a combined measurement of atherosclerosis type, extent and severity.

Microscopic evaluation of aortic atherosclerosis can be performed qualitatively or quantitatively. Different quantitative methods are used (*Clubb et al.* 1989, Fischer Hansen et al. 1992, 1994). The quantitative microscopic evaluation is a measure of severity and extent of aortic atherosclerosis.

The microscopic quantitative technique for examination of coronary arteries in rabbit heart demonstrating location, extension and severity of atherosclerotic lesions, and the validation of different methods for evaluation of atherosclerosis in the rabbit has been recently published (*Fischer Hansen et al.* 1994).

Conclusion

The rabbit was the first animal species in atherosclerosis research and it has been the most popular species in this research field during the last nine decades. It's widely use is due to the fact, that a rabbit fed atherogenic diet easily and relatively quickly develops atherosclerotic lesions which is time saving for experiments. The rabbit has been severely criticized with some justification for its limitations in this field of research.

However, the rabbit withstood the critique and achieved renewed popularity. One of the reason is the preference of using species with well characterized biology. Another is, that the shortcomings of the classical model, the cholesterol-fed rabbit, concerning the morphology of the lesions and cholesterol overload in different organs have been overcome by using the lower dose of cholesterol. The most important reason, however, is the development of new genetic variants which offer expanded possibilities to explore the relationship between lipid metabolism and development of atherosclerosis.

Summary
The rabbit was the first animal model in atherosclerosis research and it has been the most popular species during the last nine decades, despite some critique concerning the nature of the experimental atherosclerosis. The rabbit fulfils a number of practical and biological criteria for being an ideal animal model for human atherosclerosis. The shortcomings of the classical rabbit model, the cholesterol-fed rabbit, concerning the morphology of the lesions have been overcome, and new genetic variants which offer expanded possibilities of exploring the relationship between lipid metabolism and development of atherosclerosis have been developed. At present, the mostly used rabbit models are: the cholesterol-fed rabbit, the Watanabe heritable hyperlipidemic rabbit, and St. Thomas' Hospital rabbit. Different study designs can be applied to the cholesterol-fed rabbit. Atherosclerosis in rabbit models can be evaluated by macroscopic, biochemical and microscopic methods.

Sammendrag
Kaninen var den første dyremodel i atheroskleroseforskningen og den mest populære, på trods af en del kritik rettet mod karakteren af den eksperimentelle atherosklerose. Kaninen opfylder de praktiske og de biologiske kriteria, som stilles til en ideel dyremodel for human atherosklerose. Nogle svage sider ved den klassiske model, den kolesterolfodrede kanin, vedrørende morfologien af de atherosklerotiske forandringer er blevet overvundet, og nye genetiske varianter, som åbner mulighederne til at udforske sammenhængen mellem lipoproteinmetabolismen og udviklingen af atherosklerose, er blevet udviklet. De på nuværende tidspunkt mest anvendte kaninmodeller er: den kolesterolfodrede kanin, Watanabe heritable hyperlipidemic kaninen, og St. Thomas' Hospital kaninen. Atherosklerose i kaninmodeller kan evalueres ved makroskopiske, biokemiske og mikroskopiske metoder.

Yhteenveto / K. Pelkonen
Kani oli ensimmäinen ateroskleroosin eläinmalii. Se on myös viimeisten yhdeksän vuosikymmenen ajan ollut suosituin laji huolimatta kokeellisen ateroskleroosimallin luonteeseen kohdistuneesta kritiikistä. Kani täyttää lukuisia käytännölisiä ja biologisia kriteereitä. Kolesterolilla ruokitun klassisen kanimallin leesioitten morfologian puutteet on myös ylittää. On kehitetty uusia geneettisiä muunnoksia joilla on mahdollistalaajemmin tutkia rasva-aineenvaihdunnan ja ateroskleroosin kehittymisen välistä suhdetta. Tällä hetkellä yleisimpiä kanimalleja ovat kolesteroliruokittu kani, perinnöllisesti hyperlipideminen Watanabe-kani ja St. Thomas's Hospital-kani. Kolesteroliruokittua kania voidan tutkia monin eri koejärjestelyin ja ateroskleroosia voidaan arvioida makroskooppisin, biokemiallisin ja ikroskooppisin menetelmin.

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