



## Anti-Atherosclerotic Effect of Probucol in WHHL Rabbits: Are There Plasma Parameters to Evaluate This Effect? (1991)

Finckh B, Niendorf A, Rath M, Biesiegel U. *Arteriosclerosis*, 9: 579-592.

### **Summary**

Probucol has been used as a lipid-lowering agent for over 10 years. Lately it has been found that its antiatherogenic action is due mainly to its antioxidative capacity, in addition to its known lipid-lowering effect. To study the antioxidative capability of probucol and its influence on plaque development we used the animal model of the LDL-receptor-defective Watanabe heritable hyperlipidemic (WHHL) rabbit. In this study we measured all lipid values before and after probucol feeding and compared them with corresponding values in untreated controls. Probucol levels were determined, as were the physiological antioxidants alpha and gamma tocopherol (vitamin E). Thiobarbituric reactive substances were measured in plasma as a parameter for lipid peroxidation. In addition to the biochemical measurements the plaque area was analyzed macroscopically and microscopically to check the antiatherosclerotic effect and correlate it with the biochemical parameters. In four experiments we showed that probucol treatment in WHHL rabbits decreases the progression of atherosclerotic plaques by way of a combined lipid-lowering and antioxidative effect.

This article is based in part on the doctoral study performed by Barbara Finckh in the Faculty of Biology, University of Hamburg Offprint requests to: U. Beisiegel

### **Full Study**

Probucol is well known to reduce cholesterol levels in blood and to induce regression of xanthomata in patients with familial hypercholesterolemia [1]. The biochemical mechanisms responsible for the antiatherosclerotic effects of probucol are of special interest. In addition to its cholesterol-lowering effect, probucol acts as an antioxidant. As such, it is able to prevent the oxidative modification of LDL in vitro [2]. Moreover, probucol has been reported to enhance the HDL-mediated cholesterol efflux from human skin fibroblasts [3].

WHHL rabbits are characterized by a inherited LDL receptor defect. They have cholesterol levels about 20 times those of normal rabbits and develop severe arteriosclerosis at an early age with a diet of normal rabbit chow [4, 5].

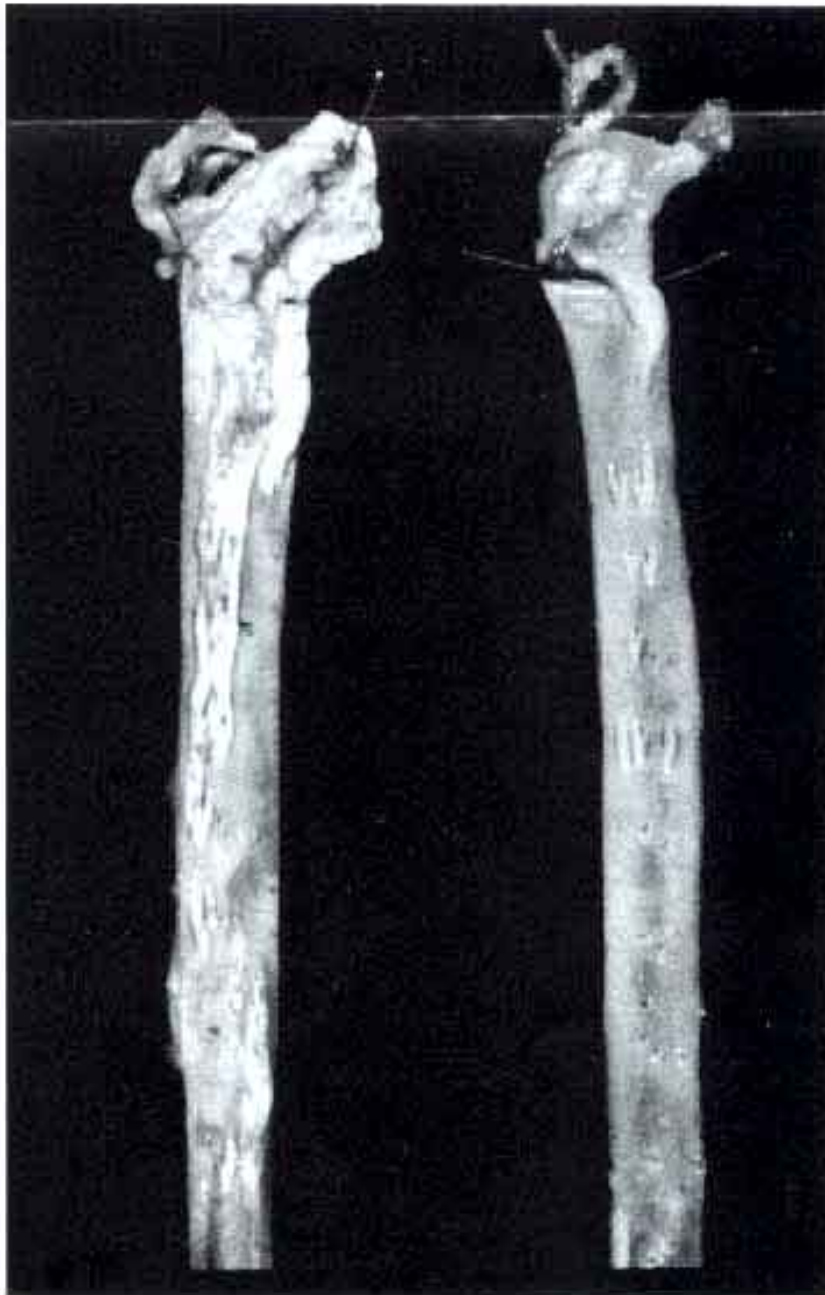


Figure 1.

**Materials and methods**

Female and male WHHL rabbits were used at different ages. In all, 22 animals were used in four experiments, and in experiments 1-3 only animals from the same litter were used in any one experiment. All rabbits were fed normal rabbit chow. The probucol (1% w/w) was mixed in this chow.

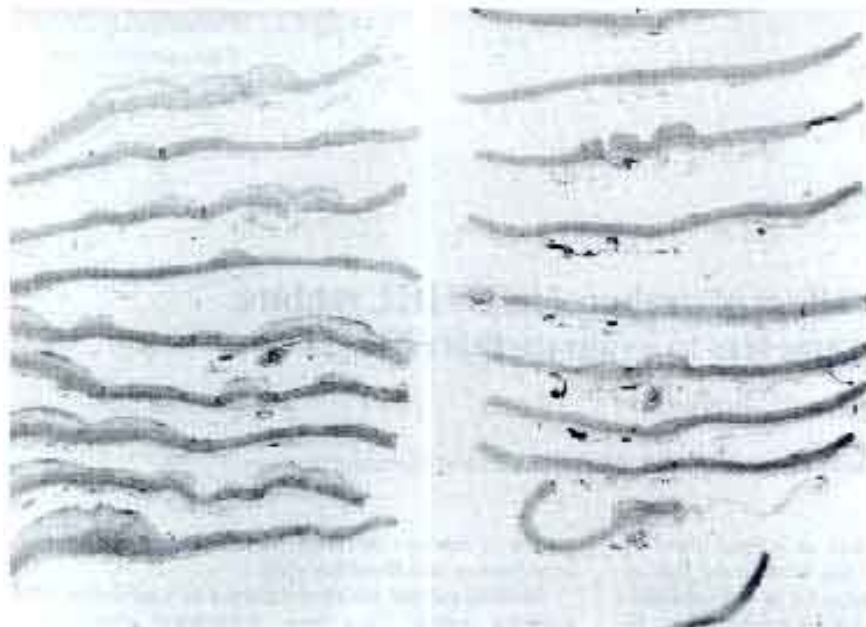


Figure 2.

Total cholesterol and total lipids were determined by standard colorimetric methods (Diagnostica, Boehringer Mannheim, FRG; Merck, Darmstadt FRG). Probucol levels and the concentration of the physiological antioxidants (alpha and gamma tocopherol) were measured by HPLC [6, 7]. Thiobarbituric acid-reactive substances (TBARS) were determined in plasma as a parameter of lipid peroxidation [8]. The plasma parameters were obtained before treatment and after the indicated treatment period.

In addition to the biochemical measurements the plaque area was analysed macroscopically and microscopically in the rabbit aortas to check the antiatherosclerotic effect and correlate it to the biochemical parameters. Macroscopically the plaque area was analysed with the aid of computer-assisted planimetry. For the histological examination the tissue was formalin-fixed and paraffin-embedded according to standard procedures. Special attention was paid to representative cross sections through the entire aortic wall. Therefore, 30 sections of each vessel were embedded in three paraffin blocks, and then cut and stained.

## Results

Four different experiments were performed with WHHL rabbits to determine the effect of probucol. Notes of age of animals at beginning and end of the experiments, length of feeding period, and sex of animals are given in Table 1. The percent plaque area measured in the aortas of the rabbits at the end of the experiment is given in Table 2. A macroscopical picture of the aorta of an untreated animal compared to a sibling treated with probucol (expt.2) is given in Fig. 1. In each of experiments 2-4 one animal was sacrificed at the beginning of the experiment and its plaque area was measured as pre-treatment control. In Fig. 2 an overview of the middle part of the aorta of a probucol-treated animal and a non-treated control is shown in low-power magnification of a histological section. It is evident that the non-treated animal has a far greater lesioned area than the probucol-treated rabbit. Histological examination reveals that (1) the major type of lesions in the control animal are complicated atheroma (i.e. lesions including necrosis and calcification) in contrast to more fibrous and generally less elevated lesions in the control rabbit; (2) the non-treated animal has more foam cells in its atheromas than the probucol-treated animal.

The mean cholesterol levels of all animals at the beginning of and during the experiment is given in Fig. 3. A 20-30% decrease in total cholesterol could be observed after 6, 12 months of treatment. The TBARS, probucol and tocopherol values in plasma are given for the treated rabbits and for the untreated controls from all experiments in Fig. 4. The probucol levels vary between 3.2 and 9.3 mg/mg total lipids. The TBARS are decreased in the treated animals. This might be an indication of a lower degree of lipid peroxidation in the blood or the arterial wall of these animals. A slight increase could be seen in the alpha tocopherol and the measurable gamma tocopherol that had appeared in the treated animals.

**Table 1. Description of the experiments**

Expt.	No. of animals	Age at start	Feeding	Age at end
1	1 female; 5 male	1 month	6 months	7 month
2	3 female; 3 male	2 months	12 months	14 month
3	3 female; 2 male	5 months	6 months	11 month
4	5 female	15 months	6 months	21 month

**Table 2. Intimal plaque area as percentage**

Expt.	Pre <sup>a</sup>	C 1	C 2	C 3	P 1	P 2	P 3
1	-	7.5	1.2	4.5	1.1	1.8	0.9
2	0	37	36	-	21	12	7
3	55	80	62	-	42	20	-
4	80	97	88	-	94	93	-

c, Animals fed normal chow throughout the experiment; p, animals fed normal chow with 1% probucol w/w

<sup>a</sup> Animals sacrificed at the beginning of the experiment as pretreatment controls

Table 1+2.

In the first experiment animals were only 1 month old when we started the feeding experiment. We therefore did not kill an animal as a pre-treatment control, since at this age we did not expect any visible plaque development. After the 6 months of the experiment only around 5% plaque area was measured in the aorta of the untreated animals. This means that this litter was less prone to the development of atherosclerotic plaques than the other animals seen in this study (see expt.3). The probucol-treated rabbits showed an even smaller percent, age plaque area (1-2%) after the 6 month period. The mean cholesterol values were 686 mg/dl in the controls and 569 mg/dl in the probucol-treated animals. Only very slight differences were observed in TBARS, but the treated animals with the lowest TBARS (0.07 nmol/mg total lipid) also had the smallest plaque area, with 0.9%.

Six 2month-old animals were used in the second experiment. No plaques were detected in the pre-treatment control. The untreated animals developed 36% and 37% plaque area in the aortas over the 12 months of the experiment (see Fig. 1). In comparison, the three probucol-treated animals developed only 7-21% plaque area. The cholesterol at the end of treatment was 715 m/dl in the controls and 515 mg/dl in the probucol-treated animals. The difference in TBARS between the treated animals and the controls was rather small (0.086 versus 0.1 nmol/mg total lipids). In this experiment probucol was able to slow down the

early plaque development in rabbits age 2-14 months.

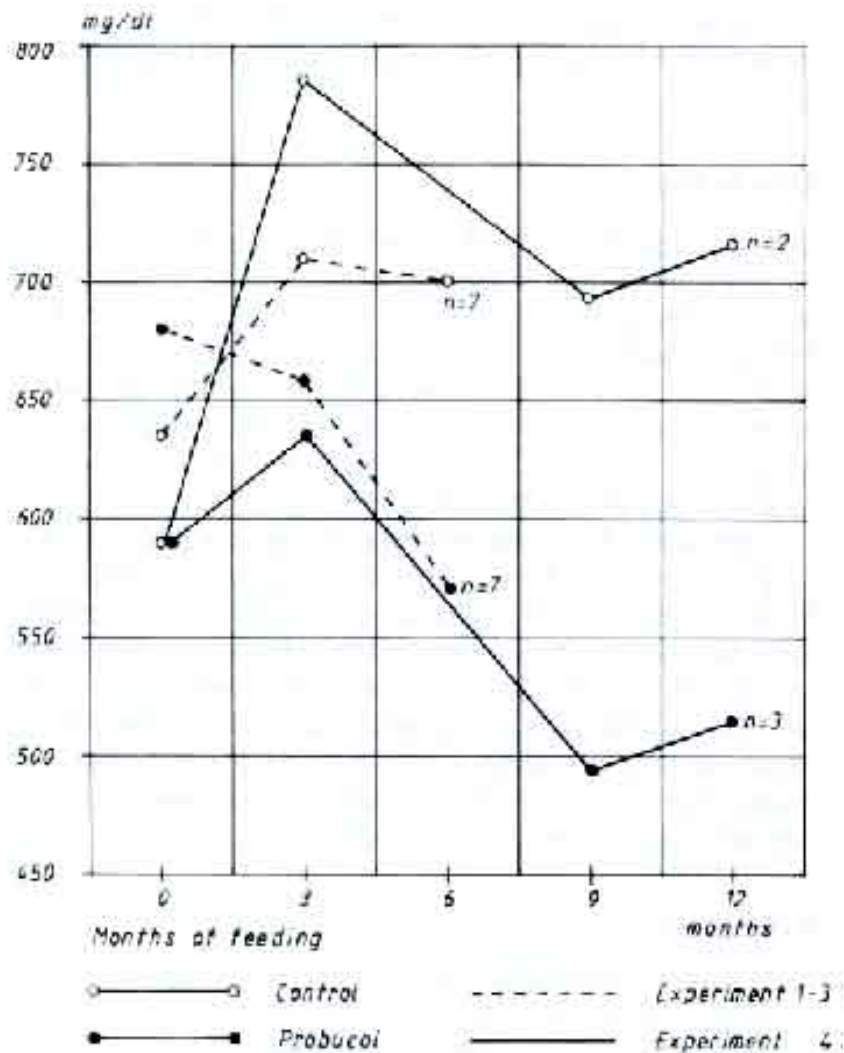


Figure 3.

In the third experiment we started the treatment at the age of 5 months. The pre-treatment control sacrificed at this age had 55% plaque area in its aorta. After the 6 months of feeding, in controls the aortic plaque area had increased to 62% and 80%, while in the treated siblings we measured only 42% and 20% plaque area, i.e. smaller areas than in the pre-treatment control. This indicates that regression of early plaques may be possible in young animals after probucol treatment. The values for lipid peroxidation in plasma, the TBARS; were 0.25 nmol/mg total lipid in controls versus 0.1 nmol/mg total lipid in the probucol-treated animals. The mean cholesterol values were 788 mg/dl in the controls versus 438 mg/dl in the treated animals. Both biochemical parameters show significant differences, which might be responsible for the regression of the plaque area seen in this experiment.

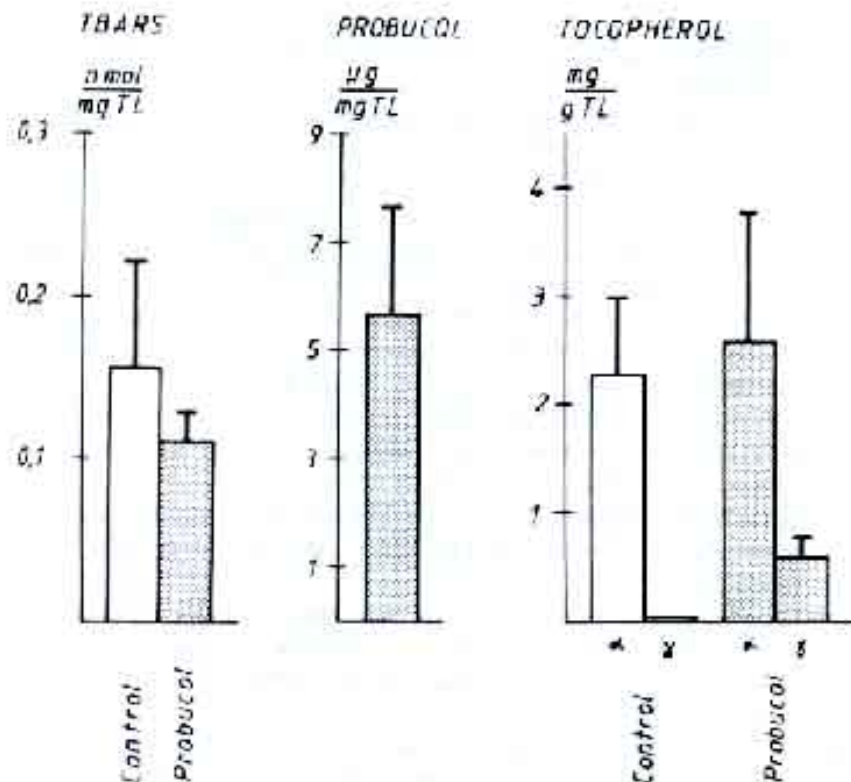


Figure 4.

The question as to whether probucol can also have an effect in very old rabbits was addressed in the fourth experiment. The 15-month-old female animals were not from the same litter but had the same parents. The three pre-treatment controls already had an average of 83% plaque area in the aortas. The probucol treatment did not have any effect on plaque development (94% versus 95%). The cholesterol was 631 mg/dl in the controls and 706 mg/dl in the treatment group. This experiment shows that in older animals probucol is not effective in cholesterol lowering and that existing severe plaques are also not affected by the treatment.

### Discussion

In this study we measured plasma lipids, antioxidants and lipid peroxidation parameters and compared these biochemical values with the plaque development in the aortic intima of rabbits. We used animals from the same litter in each of experiments 1-3 to ensure the same genetic background in the animals we wanted to compare. One interesting observation on comparison of the litters was that even though they were derived from the same colony they differed in the age of onset of plaque development. The 5-month-old rabbits in experiment 3 already had 55% plaque area, while the 7-month-old control animals in experiment 1 had only 1-8% plaque area; and the 14-month-old female in experiment 2 had 37% plaque area while the females in experiment 4, which were only 1 month older, already had around 88% plaque area. Littermates, in contrast, were more similar in the amount of plaque development at the same age. We have no explanation for this observation as yet, but we consider it makes it even more important to compare only animals from the same litter.

Probucol was able to reduce the cholesterol level significantly in younger animals, but no effect was observed in the 15-month-old female animals. The TBARS were lower in the treated animals, but the difference were not significant and we are not yet sure whether we can detect measurable differences for this parameter in human plasma of patients treated with probucol.

The increase in alpha tocopherol and the appearance of gamma tocopherol in the plasma of the treated animals confirm earlier observations that treatment with probucol preserves the physiological antioxidant tocopherol.

When the plaque area measured in the aortas of WHHL rabbits after treatment is compared with that in untreated controls it becomes obvious that probucol slows down the progression of atherosclerosis in WHHL rabbits. The histological investigation allows a more detailed analysis of the plaque composition. The most striking histological finding in the probucol-treated animals is a reduction in foam cells in the early period of life. Thus, inhibition of LDL oxidation (as performed here in an in vivo experiment) prevents the overloading of macrophages with cholesterol. This, together with stimulation of the reverse cholesterol transport, might be the basis of the antiatherogenic potential of probucol.

These data confirm observations published by Kita et al. [9] and Carew et al. [10]. In these author studies, however, the prevention of progression was not correlated to biochemical parameters for lipid peroxidation. With our study we want to contribute to the explanation of the mechanism by which probucol prevents plaque development. If this were understood it might enable us to find diagnostic parameters allowing a better estimation of the individual risk of early coronary heart disease in individual patients. Moreover, it might allow us to measure the efficiency of the treatment with antioxidants, what is not possible at present

### **Conclusion**

In four experiments with 22 WHHL rabbits we demonstrated that probucol treatment decreased the progression of atherosclerotic plaques by a combination of cholesterol lowering and antioxidative effect. In one litter we could even show a reduction of plaque area by the treatment in comparison with a sibling sacrificed at the beginning of the experiment. This indicates that regression might be possible in WHHL, rabbits. Our study demonstrates that differences in biochemical parameters for lipid peroxidation such as TBARS can be measured in probucol-treated animals versus controls and might later be used to check the efficiency of the drug treatment. In addition, the increased tocopherol level in the plasma of the animals might indicate the positive drug response. The possibility has to be evaluated whether increased levels of tocopherol can be used as a measure of antiatherogenic effects in patients.

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